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Effect of a Diet Relatively Low in Cystine on the Production of Spontaneous Mammary-Gland Tumors in Strain C3H Female Mice

By JULIUS WHITE, *senior biochemist*, and HOWARD B. ANDERVONT, *principal biologist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The effect of a diet relatively low in cystine on the incidence of leukemia induced by painting strain dba mice with an ether solution of methylcholanthrene has been reported (*1*). This report indicated that when strain dba mice were placed on a diet low in both protein and cystine a decrease in the incidence of leukemia from 89 to 17 percent resulted; sclerotic lesions of the aorta and other large arteries occurred frequently among the mice that ingested the low cystine diet. The assumption was made that cystine might be required to detoxify methylcholanthrene, and as a result the vascular damage was a manifestation of the toxicity of the methylcholanthrene, probably not related to its carcinogenic properties. It was of further interest to determine whether the reduction of the incidence of induced leukemia in strain dba mice on a diet low in cystine was specific or whether it was applicable to the prevention of the development of spontaneous mammary-gland tumors in a susceptible strain.

This paper reports the incidence of spontaneous mammary-gland tumors in a subline of strain C3H under dietary regimes identical with those employed in the study of induced leukemia. Andervont (*2*) recently found an incidence of over 97 percent spontaneous mammary-gland tumors in 350 C3H virgin females of this subline at an average age of 10.43 months. The advantages of studying the effect of a low cystine diet on the development of spontaneous mammary-gland tumors over that of induced leukemia in strain dba mice were twofold: (1) No carcinogenic agent was needed; and (2) no secondary changes such as those observed in the strain dba mice painted with methylcholanthrene could be attributed to a carcinogenic agent.

The diet used in the earlier study (*1*)

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was employed in the present study, and had the following composition:

	Percent
Casein (vitamin-free)	3. 9
Starch (corn)	55. 1
Crisco	22. 0
Cod-liver oil	3. 0
Salts (modified Osborne-Mendel)	6. 0
Ryzamin B (Burroughs-Wellcome)	5. 0
Liver extract (Lilly 343)	5. 0
	100. 0

This diet is referred to as the low cystine diet. The replacement of 0.5 percent starch by 0.5 percent *l*-cystine is referred to as the high cystine diet. The effect of these diets on the growth of several strains of mice has been reported previously (*3*).

Virgin female strain C3H litter mates at the time of weaning (4-5 weeks) were divided into two groups. One group was placed on the low cystine diet, and the other on the high cystine diet. The mice were kept two in a cage, and body weight and food consumed were determined semiweekly. At each weighing the mice were examined for mammary-gland tumors. When such a tumor was observed, a record was made, and the animal allowed to live for several weeks. All animals were necropsied; from those bearing tumors, sections were prepared and the diagnosis of mammary-gland tumors was made by histologic examination.

RESULTS AND DISCUSSION

The results are summarized in table 1. The data represent a composite of two series of experiments. The second series was started before the first one had been terminated. In the table are included, for comparison, Andervont's data (*2*) on the occurrence of spontaneous mammary-gland tumors in virgin strain C3H mice. These mice were maintained on a Purina dog chow diet, the composition of which has been given previously (*2*). The mice ingesting the high cystine diet showed an incidence of mammary-gland-tumor formation almost identical with that of the animals on the dog chow diet. The remarkable difference in tumor incidence between the animals ingesting the low cystine and the high cystine diet is noteworthy. Even at the end of 22 months, none of the animals on the low cystine diet developed spontaneous mammary tumors. Mice on the low cystine diet were much smaller and at the age of 6 months weighed an average of 12.4 gm. as compared with 25.3 gm. for the animals on the high cystine diet at the same age. However, examination of food intake indicates that the low cystine group consumed an amount of food equivalent to that consumed by the high cystine group if the food intake is based on a unit of body weight. On the

TABLE 1.—*Appearance of spontaneous mammary tumors or of death in strain C3H virgin female mice*

[Numerators = number of mice developing tumors
Denominators = number of mice dying free from tumor]

Diet	Number of mice	Mice developing tumors or dying free from tumor according to age (in months)																Tumor incidence	Average tumor age
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	Total		
																		Percent	*Months*
Chow	[1] 350	3/0	11/1	20/0	35/0	63/0	51/0	56/1	41/1	26/1	13/1	10/2	5/0	6/0	1/1	0/1	341/9	97. 43	10. 43
High cystine	42	0/0	11/1	5/0	9/0	6/0	2/0	4/0	3/0	1/0	.....	.....	.....	.....	.....	.....	41/1	97. 61	8. 36
Low cystine	45	0/0	0/0	0/0	0/0	0/0	0/0	0/3	0/2	0/0	0/1	0/0	0/2	0/1	0/0	0/0	0/9	.00	.....

[1] Data from Andervont (*2*).

basis of food intake, then, this difference in mammary-tumor development cannot be attributed to a decrease in caloric consumption. Morris and Lippincott (*4*) have made a similar observation with regard to caloric intake and mammary-tumor growth.

The mice on the low cystine diet had irregular oestrous cycles, or, in some cases, oestrus was absent. The possible association of the irregularity or absence of oestrus with the failure of mammary-gland tumors to appear in the mice in the low cystine group must not be overlooked. Gardner (*5*) pointed out that usually it has been impossible to associate the oestrous cycle with the tendency to tumor development. However, dietary factors may alter the oestrous cycle somewhat, according to Visscher, Ball, Barnes, and Sivertsen (*6*). In the work of these authors, as well as in the data reported here, there was an interruption in the regularity of oestrus as well as a failure to produce tumors.

Gross mounts of breast tissue were made from six of the mice on the low cystine diet at ages varying from 11 to 21 months. These mounts showed the characteristic breast-duct acinar pattern of a low-mammary-tumor strain described by Taylor and Waltman (*7*). Similar mounts from the high-cystine-diet mice from 9 to 11 months of age showed the acinar pattern characteristic of a high-mammary-tumor strain of mice.

SUMMARY AND CONCLUSIONS

Litter-mate virgin strain C3H mice at the time of weaning were divided into two groups: one was placed on a relatively low cystine diet and the other on a high cystine diet.

The mice on the low cystine diet failed to develop spontaneous mammary-gland tumors, even at the end of 22 months. The mice on the high cystine diet developed tumors to the same extent and in about the same average time as did those of the same strain on a dog chow diet.

Mice on the low cystine diet showed irregular, and in many cases complete absence of, oestrous cycles. This may be associated with the failure to develop mammary-gland tumors.

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Note on the Comparison of Dosage of Methylcholanthrene on the Production of Leukemia and Sclerotic Lesions in Strain Dilute Brown Mice on a Restricted Cystine Diet

By JULIUS WHITE, *senior biochemist*, G. BURROUGHS MIDER,[1] and WALTER E. HESTON, *geneticist, National Cancer Institute, National Institute of Health, United States Public Health Service*

In a previous paper (*1*), it was reported that the incidence of leukemia induced in methylcholanthrene-painted mice of the Dilute Brown (dba) strain was reduced when the concentration of cystine in the diet (*2*) was decreased to a level which permitted maintenance of weight but not weight increment in young animals. It was also reported that sclerotic lesions of the aorta and other large arteries occurred frequently among the mice which ingested the low cystine diet. The suggestion was made that the vascular damage was a manifestation of the toxicity of the methylcholanthrene and was probably not related to its carcinogenic properties.

In previous experiments mice were painted with a 0.2-percent solution of methylcholanthrene in ether on alternate days until they were dying. The site of application was changed at each painting, according to the technique of previous investigations (*1*, *3*). Usually 40–70 paintings (average 44.7) were made. The amount of methylcholanthrene exceeded that needed to produce leukemia, and the excess probably produced toxic symptoms as evidenced by the appearance of sclerotic lesions.

This paper deals with the effect of the application of a limited amount of methylcholanthrene on the incidence of leukemia and sclerotic lesions in dba mice ingesting a high or a low cystine diet.

In a series of preliminary experiments,[2] several groups of dba mice were placed on the Purina dog chow diet and given 10, 15, 20, or 30 paintings with methylcholanthrene. The technique referred to previously was used. In this way it was possible to determine the least amount of methylcholanthrene necessary for the production of leukemia in most of the mice. When the animals were painted 20 times, the incidence of leukemia was nearly equivalent to that obtained from 40–70 paintings.

In this work, litter mates of Dilute Brown mice of the subline 212 after weaning were divided into three groups. One group was fed Purina dog chow, another the low cystine diet, and the third the high cystine diet (*2*). All the mice were painted (*1*) 20 times on alternate days with a 0.2-percent solution of methylcholanthrene in ether and then observed until death.

The results are presented in table 1. Included in the table are data from previous work (*1*) on the effect of an average of 44.7 applications of methylcholanthrene

[1] *Now at Department of Pathology, Cornell Medical College.*

[2] *Unpublished data.*

on two groups of mice ingesting the high or the low cystine diet, respectively.

Inspection of the table reveals that the incidence of leukemia in animals painted 20 times and ingesting either the chow or the high cystine diet was essentially the same as that for animals that received 40–70 applications of methylcholanthrene. The mean latent period, however, was somewhat longer. The incidence of sclerosis of the aorta was a little lower but not significantly so, while the latent period was lengthened markedly. Although sclerosis was not prevented by decreasing the amount of methylcholanthrene, nevertheless the animals survived longer than did those on the high cystine diet that developed leukemia. In other words, by decreasing the number of paintings (i. e., total dose of methylcholanthrene) it was possible to prolong the life of the animals on the low cystine diet so that they actually outlived the animals on the high cystine diet but still failed to develop leukemia. It appears, therefore, that the sclerotic lesions of the aorta are associated with the toxic nature of the methylcholanthrene.

TABLE 1.—*Effect of diets on response of strain Dilute Brown mice to methylcholanthrene* [1]

Diet	Number of mice	Mice with—					
		Leukemia [2]			Sclerosis of aorta [3]		
		Number	Percent	Mean latent period	Number	Percent	Mean latent period
40-70 PAINTINGS							
				Days			*Days*
Dog chow	19	17	89.4	81	0		
High cystine	39	35	89.8	81	1	2.6	92
Low cystine	35	6	17.1	91	33	94.2	80
20 PAINTINGS							
Dog chow	40	36	90.0	99	0		
High cystine	40	36	90.0	101	0		
Low cystine	40	4	10.0	127	32	80.0	114

[1] Data on mice receiving 40–70 paintings (mean, 44.7) taken from White and Mider (*1*).
[2] Pathologic diagnosis described by Mider and Morton (*3*).
[3] Pathologic diagnosis described by White and Mider (*1*).

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Quantitative Experiments on the Production of Subcutaneous Tumors in Strain A Mice with Marginal Doses of 3, 4-Benzpyrene [1]

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INTRODUCTION

For more than a decade the production of tumors with chemical agents in experimental animals has been studied by numerous investigators. While a large amount of interesting data has been accumulated, little information is available with regard to the mechanism of carcinogenesis by such agents, as was pointed out previously (*2*).

It had been found in this laboratory that the basic fraction of creosote oil promoted tumor production with marginal doses of 3,4-benzpyrene both in skin-painting (*3, 4*) and in subcutaneous-injection experiments (*4*). This raised the question whether substances of biologic origin might be found to exert either a promoting or retarding influence on the action of carcinogens. Accordingly, the experiments described in the present paper were carried out during the years 1938–42.

In much of the published work with carcinogens, doses were selected for production of the maximum number of tumors in the shortest time. However, earlier work (*3, 4*) in this laboratory supported the viewpoint that factors which influence tumor production might be obscured when overwhelming doses of carcinogen were employed, a possibility which has been discussed by several writers (*5–8*). Consequently, experiments were carried out to ascertain the range of dose levels that would be suitable for experiments designed to reveal the influence of promoting or retarding factors. Low doses of 3,4-benzpyrene were injected subcutaneously into mice to determine the marginal range, i. e., doses large enough to induce tumors regularly in a substantial proportion of the mice but not so large as to produce tumors in all the animals.

When the present study was begun, there was little information available on the response of inbred strains of mice to the carcinogenic action of polycyclic hydrocarbons in doses below 0.2 mg. Since then Tannenbaum (*8–10*) has reported results obtained with doses of this magnitude in studies of the effect of diet on tumor genesis. Bryan and Shimkin (*11*) have attempted to place the dose-response relationship in experimental carcinogenesis with polycyclic hydrocarbons on a quantitative basis. However, there are as yet no data available on the *reproducibility* of tumor production with low doses of carcinogenic compounds under standard conditions. Blum, Grady, and Kirby-Smith (*12, 13*) have studied the limits of accuracy of tumor induction with ultraviolet radiation and have considered the accuracy to be expected when experimental groups are compared, but

[1] *Presented* (1) *in part at the Chicago meeting of the American Society of Biological Chemists, April 1941.*

it is not as yet known how applicable the findings with marginal doses of ultraviolet radiation are to those obtained with marginal doses of hydrocarbon carcinogens.

This paper records the results of 108 experiments in which 4,814 strain A mice of both sexes received, subcutaneously, doses of benzpyrene ranging from 0.2 to 0.05 mg. The purpose was to develop a standard technique which would yield results of satisfactory reliability in control groups so that a sound basis would be available for comparison with results of experiments in which promoting or retarding factors were studied. A number of solvents (lard, cetane, tributyrin, and tricaprylin) were examined for their suitability as vehicles for this purpose. Exploratory experiments were carried out with a large number of substances of biologic origin in an attempt to uncover leads on promoting or retarding agents.

However, the problem of developing a standard technique which would give results of the desired reliability in the control groups was found to assume such proportions as to necessitate far more work than had been anticipated. Accordingly, this paper deals chiefly with the question of a reproducible standard technique for tumor production by low doses of benzpyrene.

MATERIALS AND METHODS

The samples of 3, 4-benzpyrene were purified specimens with melting points between 178.2° and 179.0° C. (corrected). The lard used as vehicle was obtained from specimens of good-quality, pure leaf lard purchased on the open market. The lard was filtered at 38° for 24 to 36 hours through coarse filter paper; less than half passed through. The fraction which was liquid at 38° and which passed through the paper was designated "lard filtrate"; the material remaining on the filter paper was designated "lard residue." Both the filtrate and the residue were employed directly; they were also further separated into fractions of different degrees of unsaturation, and into their free fatty acid, neutral fat, and unsaponifiable components. Synthetic tricaprylin was purified by various techniques including distillation at 1-mm. pressure, molecular distillation,[2] and recrystallization. The starting materials for the synthesis of the various specimens of tricaprylin, which were prepared by Hartwell (*14*), included *n*-caprylic acid (Eastman Kodak No. 665), Neo-Fat No. 7 (a commercial grade of *n*-caprylic acid), and its acid chloride. The two latter materials were obtained from Armour & Co. Commercial specimens of tributyrin (Eastman Kodak No. 726) and of cetane ($C_{16}H_{34}$), a du Pont product (boiling point 284.3°–287.3° C.; melting point 16.6°) were used without further purification. The basic fraction of creosote oil was the same preparation as that used previously (*3, 4*). A number of the other basic fractions of coal tar were obtained from the Barrett Co. The other triglycerides used were tristearin (Eastman Kodak No. 1380) with a melting point of 70°–71°, and tripalmitin (Eastman Kodak No. 1236) with a melting point of 64.5°–65.5°. They were used without further purification.

All the animals were strain A mice obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. In most cases 50 mice, equally divided between the sexes, were employed in each experiment; in a few of the earlier ones 20 to 30 animals were used. The mice were less than 6 months old at the be-

[2] *This distillation was carried out in a cyclic still manufactured by the Distillation Products Inc. and located in the Division of Agricultural Chemical Research, Bureau of Agricultural Chemistry, U. S. Department of Agriculture, Washington, D. C.*

ginning of the experiments. Purina dog chow and tap water were freely available at all times.

The animals were given a single subcutaneous injection of 0.2 cc. of solution into the left flank. Since these oily vehicles had a high coefficient of expansion, the solutions were made up to volume at 38° C. and kept at that temperature during the injection. In the case of lard residue, which was not fluid at 38°, the experiments were performed at the slightly higher temperature required to liquefy it. Added materials were either dissolved or suspended in the solution. The animals were examined by palpation at appropriate intervals for progressively growing masses.

Tumors arising at sites other than that of injection were not included in the tabulations of induced tumors. The diagnosis of tumor was made on the basis described in a previous paper (*15*).

In the tables, the plus signs refer to the total number of mice in which tumors had been induced at the site of injection at various periods, while the minus signs refer to the negative survivors at these times; the data are expressed in percent. As in previous usage (*15*), the word "negative" denotes mice not bearing induced subcutaneous tumors. Since survival was satisfactory in most experiments, the percentages are given on the basis of the initial number of mice rather than on the effective total.

EXPERIMENTAL DATA

Lard Filtrate; Reproducibility with the Same Specimen

Table 1 summarizes the results with injections of a single dose of 0.1 or of 0.065 mg. of benzpyrene in solution in 0.2 cc. of lard filtrate. The same specimen of lard filtrate was the vehicle in all four experiments.

In the early months of these experiments, more tumors were produced with 0.1 mg. of the carcinogen than with 0.065 mg. However, by 4 months there was little difference, and by 6 months similarly high results were obtained at both dose levels. These doses gave higher yields of tumors than had been anticipated on the basis of earlier work (*4*), in which 0.1 mg. of benzpyrene dissolved in lard filtrate elicited tumors in only 30 percent of the mice at 7 months. In the present experiments, 0.1 mg. produced tumors in 84 and 94 percent, respectively, of the mice in 6 months. Even more unexpected was the high yield obtained with the lower dose of 0.065 mg.

In these two sets of experiments, in which the duplicates were carried out at different times, the same doses of benzpyrene in the same specimen of lard filtrate gave results of excellent reproducibility.

Lard Residue; Reproducibility with the Same Specimen

The fraction of lard retained on the filter paper at 38° C. was also employed as vehicle. Table 1 gives the results obtained in three experiments, performed at different times, with 0.1 mg. of benzpyrene in solution in the same specimen of lard residue. Tumor production was strikingly lower than when lard filtrate was used as the vehicle for 0.1 mg. of the carcinogen. As before, the reproducibility was satisfactory. At 9 months the percentages represent tumor production in 3, 5, and 7 mice, respectively, of an initial number of 50.

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TABLE 1.—*Reproducibility of production of subcutaneous tumors with the same sample of lard as vehicle for 3,4-benzpyrene*

Experiment No.	Benzpyrene injected	Mice [1] with tumors (+), and negative survivors (−), after various periods		
		4 months	6 months	9 months
	Milligrams	*Percent*	*Percent*	*Percent*
LARD FILTRATE				
38a	0.1	72+ 22−	84+ 6−	86+ 2−
38b	.1	88+ 8−	94+ 2−	94+ 2−
43	.065	72+ 28−	84+ 16−	84+ 16−
98	.065	62+ 36−	80+ 18−	84+ 8−
LARD RESIDUE				
39a	.1	0+ 90−	4+ 78−	6+ 66−
39b	.1	0+ 100−	4+ 94−	10+ 76−
100	.1	2+ 98−	4+ 84−	14+ 40−
44 [2]	.065	0+ 86−	4+ 70−	4+ 62−

[1] 50 mice, equally divided between the sexes, used in each experiment.
[2] For comparison, data obtained in a single experiment with a lower dose are included here.

VARIATION IN RESULTS WITH DIFFERENT SPECIMENS OF LARD FILTRATE

Table 2 summarizes the results of five experiments in which the same dose of benzpyrene (0.1 mg.) was injected. The factor that was varied was the specimen of lard filtrate employed as vehicle. Experiment S–1 gives, in percentages, the data obtained in a previous experiment in this aboratory (*4, table 1, experiment C, control group*). Experiments 38a and 38b, using another specimen of lard, are discussed in the section on lard filtrate. Two other experiments, not previously mentioned, were performed with two other specimens of lard.

TABLE 2.—*Variation in production of subcutaneous tumors in mice with 0.1 mg. of 3,4-benzpyrene in different specimens of lard filtrate as vehicle*

Experiment No.	Specimen of filtered lard used	Number of mice [1]	Mice with tumors (+), and negative survivors (−), after various periods		
			4 months	6 months	9 months
			Percent	*Percent*	*Percent*
S–1	A	20	5+ 95−	25+ 75−	30+ 65−
S–2	B	20	85+ 15−	95+ 5−	100+ 0−
20	C	40	30+ 70−	55+ 40−	60+ 35−
38a	D	50	72+ 22−	84+ 6−	86+ 2−
38b	D	50	88+ 8−	94+ 2−	94+ 2−

[1] The mice were equally divided between the sexes, except in experiment S–1 in which only male mice were used (*4, table 1, experiment C, control group*).

Tumor production at the end of 9 months varied widely. Good reproducibility was obtained in the two experiments (38a and 38b) in which the same lard filtrate was employed. In all experiments but one, mice of both sexes were used; in S–1, only males had been employed. This sex difference, however, cannot account for the low tumor production in experiment S–1 as compared with the results of the other four experiments, inasmuch as it has been found (see section, Effect of Sex, p. 468) that in such experiments tumors are induced more readily in males. Since only one mouse died without tumor in 9 months in this experiment, the low tumor yield is not attributable to poor survival.

At the present time it would appear that these large differences in tumor production may be owing, in part at least, to the influence of the vehicle, which was the only factor varied intentionally in this set of experiments.

Inasmuch as such experiments require the greater part of a year for completion,

exploratory attempts were made to obtain, concurrently, some information on the possible promoting or retarding influence of biologically occurring substances. By the time the data in table 2 had been accumulated, it became apparent that the variation in the results obtained with different batches of lard filtrate, in experiments in which no additional material had been incorporated into the benzpyrene-lard solution, was greater than was considered desirable for control groups in a standardized procedure. In fact, the over-all yield of tumors and the rate of tumor production were so great in some of these experiments as to raise the question whether lard filtrate itself contained a promoting substance which varied in amount from specimen to specimen.

In subsequent sections the influence of various fractions of lard upon tumor genesis is considered, together with a brief account of the experiments in which other substances were incorporated into the benzpyrene-lard solutions. Because of the variable results obtained in the control groups when different batches of lard were employed, other materials were investigated for their suitability as vehicles.

Tricaprylin as Vehicle

Fieser (*16*) stated that a pure saturated glyceride might offer advantages over lard and suggested several, including tricaprylin, that were available commercially. He also stated that slightly yellow samples could be purified by distillation in high vacuum and that the pure gylcerides were colorless, odorless, and tasteless. Accordingly, tricaprylin was employed as a vehicle for carcinogens by Shimkin and Andervont (*17*, *18*), who reported that it was excellent for this purpose. It has subsequently been used as a solvent by other workers, and it has been recommended by Shimkin (*19*) for employment in a standard technique for obtaining quantitative data in the biologic testing of carcinogens.

Tricaprylin was also used as the vehicle for marginal doses of benzpyrene in this study. Table 3 summarizes the data obtained in 12 experiments in which benzpyrene dissolved in tricaprylin was given in doses ranging from 0.05 to 0.2 mg. The four specimens of tricaprylin used were obtained from different batches of tricaprylin which were synthesized by Hartwell (*14*). Specimen A was redistilled in a molecular still in the Converse Chemical Laboratory, Harvard University, through the kindness of Professor Fieser; the melting point of this pale yellow sample was 6°–7° C. Another batch was redistilled at 1-mm. pressure, and then recrystallized without solvent; the crystals melted at 8.6°–9.2°. This specimen (B) was an almost colorless preparation. The mother liquor, which was yellow, melted at 0°–3°; it was designated specimen C. A still different batch was subjected to molecular distillation as described in the section Materials and Methods; specimen D was the fraction with the highest melting point (8.2°–9.2°). According to Dr. Hartwell *n*-caprylic acid (Eastman Kodak No. 665) was employed in the synthesis of specimen A, Neo-Fat No. 7 was used in the synthesis of specimens B and C, and the acid chloride of Neo-Fat No. 7 served as the starting material in the preparation of specimen D.

The data in table 3 show that, with any given specimen of tricaprylin as solvent, the yield of tumors induced at the injection site decreased progressively as the dose of benzpyrene was decreased from 0.2 to 0.05 mg. Similarly consistent results were obtained when all the data at different dose levels of benzpyrene were summarized from 15 experiments in which 7 different

specimens of tricaprylin were employed (table 4). The only apparent discrepancy is the relatively high value obtained with 0.08 mg. benzpyrene at 4 months. However, entirely consistent results were obtained at subsequent periods with this dose.

From these data it is evident that the dose range given in table 4 is appropriate for experiments with marginal doses of benzpyrene when tricaprylin is the vehicle. For experiments designed to ascertain promoting effects the lower end of the range, in which tumors were obtained in about a third of the mice, is considered most suitable; for studying retarding factors, the upper end of this range, in which tumors were produced in about two-thirds of the mice, is considered most suitable. In most of the experiments that were carried out with tricaprylin as vehicle, 0.065 mg. benzpyrene was employed. This dose resulted, on the average, in tumor production in about half of the animals.

The phrase "on the average" needs to be stressed. The values given in table 4 represent the arithmetic means of the values obtained in two to five experiments each, with the exception of the data for 0.08 mg. Only a single experiment was carried out at this dose level, and it is of interest that this is the only dose in which the results were not consistent at all time intervals with those obtained at other doses.

The importance of performing multiple experiments to secure reliable results is further brought out in table 5 which gives the results of the individual experiments from which the average values given in table 4 were obtained. This table contains the data secured with three specimens (E, F, G) of tricaprylin in addition to those with the four specimens recorded in table 3. Specimens E, F, and G were also synthetic preparations made by Hartwell; they had been redistilled at 1-mm. pressure. The melting points are

TABLE 3.—*Production of subcutaneous tumors in mice with different dose levels of 3,4-benzpyrene in the same specimens of tricaprylin as vehicle*

Experiment No.	Tricaprylin employed	Benzpyrene injected	Number of mice [1]	Mice with tumors (+), and negative survivors (−), after various periods			
				4 months	6 months	9 months	12 months
		Mg.		*Pct.*	*Pct.*	*Pct.*	*Pct.*
L-14	Specimen A	0.1	24	33+ 67−	67+ 33−	71+ 17−	71+ 0−
L-15	do	.05	23	13+ 83−	30+ 66−	47+ 44−	47+ 34−
L-23	Specimen B	.2	60	45+ 55−	68+ 30−	80+ 15−	82+ 3−
L-22	do	.1	60	12+ 85−	63+ 33−	75+ 20−	75+ 8−
L-21	do	.065	60	3+ 97−	32+ 66−	50+ 37−	53+ 10−
E-6	do	.05	48	4+ 96−	21+ 70−	38+ 40−	42+ 20−
E-10	do	.05	50	2+ 98−	24+ 76−	24+ 66−	24+ 24−
L-26	Specimen C	.2	60	23+ 73−	45+ 47−	53+ 30−	53+ 3−
L-25	do	.1	60	3+ 92−	37+ 55−	47+ 37−	49+ 13−
L-24	do	.065	60	3+ 95−	28+ 70−	37+ 50−	43+ 28−
86	Specimen D	.1	50	28+ 66−	58+ 36−	66+ 16−	.....
85	do	.065	50	14+ 84−	58+ 38−	60+ 28−	.....

[1] Mice equally divided between the sexes.

TABLE 4.—*Average rate of subcutaneous tumor production in mice with different dose levels of 3,4-benzpyrene in seven specimens of tricaprylin as vehicle*

Benzpyrene injected (milligram)	Number of mice [1]	Mice with induced tumors after various periods			
		4 months	6 months	9 months	12 months
		Pct.	*Pct.*	*Pct.*	*Pct.*
0.2	120	34	57	67	68
0.1	194	19	56	65	65
0.08 [2]	50	32	52	54	56
0.065	270	5	32	43	45
0.05	121	6	25	36	38

[1] Mice equally divided between the sexes.
[2] A single experiment was performed at this dose level.

given in table 5 except for specimen F, which was not recorded.

TABLE 5.—*Variation in rate of production of subcutaneous tumors in mice with the same dose levels of 3, 4-benzpyrene in different specimens of tricaprylin as vehicle.*

Experiment No.	Tricaprylin used		Benzpyrene injected	Number of mice [1]	Mice with tumors (+), and negative survivors (−), after various periods			
	Sample	Melting point			4 months	6 months	9 months	12 months
		°C.	*Mg.*		*Pct.*	*Pct.*	*Pct.*	*Pct.*
L-23	B	8.6–9.2	0.2	60	45+ 55−	68+ 30−	80+ 15−	82+ 3−
L-26	C	0.0–3.0	.2	60	23+ 73−	45+ 47−	53+ 30−	53+ 3−
L-14	A	6.0–7.0	.1	42	33+ 67−	67+ 33−	71+ 17−	71+ 0−
L-22	B	8.6–9.2	.1	60	12+ 85−	63+ 33−	75+ 20−	75+ 8−
L-25	C	0.0–3.0	.1	60	3+ 92−	37+ 55−	47+ 37−	49+ 13−
86	D	8.2–9.2	.1	50	28+ 66−	58+ 36−	66+ 16−	.....
30	G	5.0–6.0	.08	50	32+ 66−	52+ 46−	54+ 42−	56+ 34−
L-21	B	8.6–9.2	.065	60	3+ 97−	32+ 66−	50+ 37−	53+ 3−
L-24	C	0.0–3.0	.065	60	3+ 95−	28+ 70−	37+ 50−	43+ 28−
85	D	8.2–9.2	.065	50	14+ 84−	58+ 36−	60+ 28−	.....
46	E	8.3–9.0	.065	50	4+ 88−	32+ 54−	44+ 32−	46+ 12−
74	F	.....	.065	50	0+ 100−	10+ 80−	22+ 36−	24+ 18−
L-15	A	6.0–7.0	.05	23	13+ 83−	30+ 66−	47+ 44−	47+ 34−
E-6 [2]	B	8.6–9.2	.05	48	4+ 96−	21+ 79−	38+ 40−	42+ 20−
E-10 [2]	B	8.6–9.2	.05	50	2+ 98−	24+ 76−	24+ 66−	24+ 24−

[1] Mice equally divided between the sexes.
[2] From unpublished work of Engel, Leiter, and Shear (1942).

Table 5 shows that rather wide variations in tumor production were obtained at any given dose level. These variations were great enough to result in overlapping. One possible explanation for this might be the difference, from batch to batch, in the influence of the solvent. This is supported by the fact that when the mother liquor (specimen C) was employed as vehicle in experiments L–26 and L–25, tumor production was lower than in other experiments with 0.2 and 0.1 mg. benzpyrene in which preparations with higher melting points were used. However, with lower doses of benzpyrene, this correlation between tumor production and melting point was not evident. For example, at 9 months with 0.065 mg. benzpyrene, a higher yield of tumors (60 percent) was obtained in experiment 85 than in experiment 46 (44 percent) although the melting points of the two specimens of tricaprylin were almost identical. On the other hand, the value of 37 percent obtained in experiment L–24 was not much different from that of 44 percent in experiment 46 although the melting points (0.0°–3.0° and 8.3°–9.0°) of these two specimens of tricaprylin varied widely. Similarly, with 0.05 mg. benzpyrene, the tumor incidence obtained in experiment L–15 was greater than that in experiment E–6 although the melting point of the tricaprylin used in the former experiment was about 2.5° lower. Furthermore, in experiments E–6 and E–10, in which the same specimen of tricaprylin was employed, rather wide differences in tumor production were obtained after the first 6 months.

Table 6 gives the reproducibility of the tumor incidence in experiments in which the same sample of vehicle was employed. With 0.2 mg. benzpyrene good reproducibility was obtained. With 0.05 mg., however, the reproducibility was less satisfactory. This again emphasizes the necessity of having an adequate number of experiments for the reliable determination of dose-response values even when as many as 50 mice are employed in each experiment.

TABLE 6.—*Reproducibility of production of subcutaneous tumors in mice with the same sample of tricaprylin as vehicle for 3, 4-benzpyrene*

Experiment No.	Benzpyrene injected	Number of mice [1]	Mice with tumors (+) and negative survivors (−), after various periods			
			4 months	6 months	9 months	12 months
	Mg.		*Pct.*	*Pct.*	*Pct.*	*Pct.*
L-23	0.2	60	45+ 55−	68+ 30−	80+ 15−	82+ 3−
P-1	.2	138	49+ 49−	73+ 10−	.....	.....
E-6	.05	48	4+ 96−	21+ 79−	38+ 40−	42+ 20−
E-10	.05	50	2+ 98−	24+ 76−	24+ 66−	24+ 24−

[1] The mice were equally divided between the sexes except in experiment P-1 in which 53 males and 85 females were used.

MISCELLANEOUS SOLVENTS AS VEHICLES

A few exploratory experiments were carried out with three other materials as vehicles for benzpyrene. The results are given in table 7.

With tributyrin (Eastman Kodak No. 726), consistent results were obtained with four doses of benzpyrene ranging from 1.0 to 0.05 mg. Tumor production at 9 months decreased progressively. Nevertheless, this solvent was considered unsatisfactory for this investigation since tributyrin, both with and without benzpyrene in solution, produced ulceration at the site of injection in strain A mice.

With cetane ($C_{16}H_{34}$, an aliphatic hydrocarbon obtained from the duPont Co., melting point 16.6° C., boiling point 284.3°–287.3°), very low tumor production was obtained even when 0.1 mg. benzpyrene was employed.

For comparison, the results obtained in experiment E–11, taken from another investigation carried out in collaboration with Dr. Lewis Engel, are also included in table 7. In this experiment, a refined, deodorized specimen of edible sesame oil was used. Tumor production with 0.05 mg. benzpyrene was as great in 9 months as when 20 times that dose was given in solution in tributyrin.

TABLE 7.—*Production of subcutaneous tumors in mice with 3,4-benzpyrene in miscellaneous substances as vehicles*

Experiment No.	Vehicle	Benzpyrene injected	Number of mice [1]	Mice with tumors (+), and negative survivors (−), after various periods		
				4 months	6 months	9 months
		Mg.		*Pct.*	*Pct.*	*Pct.*
L-10	Tributyrin	1.0	20	55+ 45−	65+ 35−	70+ 30−
	do	.5	20	40+ 60−	55+ 45−	55+ 40−
	do	.1	20	10+ 90−	20+ 80−	20+ 60−
	do	.05	20	10+ 90−	10+ 85−	10+ 75−
L-12	Cetane	.1	24	4+ 92−	4+ 67−	4+ 17−
L-43	do	.05	24	0+ 96−	0+ 83−	0+ 61−
E-11 [2]	Sesame oil	.05	50	44+ 54−	64+ 32−	68+ 28−

[1] Mice equally divided between the sexes.
[2] From unpublished experiments of Engel, Leiter, and Shear (1942).

EFFECT OF LARD FRACTIONS

When it was found that the variations obtained with different batches of lard filtrate were so great as to make advisable the selection of a more uniform substance as a standard vehicle, the question was raised whether lard contains components which facilitate or retard the carcinogenic action of benzpyrene (*20, p. 227*).

Accordingly, lard was fractionated in two ways. By crystallization from acetone according to Hilditch (*21*) four fractions were obtained which differed in degree of unsaturation. The characteristics of the fractions obtained from both the filtrate and the residue of lard are given in table 8. On comparing the iodine values with that of triolein (a typical fat in which all three

fatty acid groups are unsaturated), of dioleostearin (with two of the three acid groups unsaturated), of oleodistearin (with one unsaturated fatty acid group), and of tristearin (no unsaturated fatty acid groups), it is seen that fractions I F and I R are the most unsaturated, and fraction IV R the least unsaturated. The amount of IV F obtained was insufficient for the determination of the iodine value; the degree of unsaturation of this fraction, however, was presumably similar to that of IV R.

TABLE 8.—*Characteristics of lard fractions prepared by crystallization from acetone*

Fraction	Content	Iodine value [1]
	Percent	
Lard filtrate (38)	100	63
I F	55	74
II F	9.5	68
III F	33.5	34
IV F	.5	
Lard residue (39)	100	57
I R	53	74
II R	6.5	70
III R	31	35
IV R	10	14

[1] For comparison, the iodine values of various mixed triglycerides are: triolein, 87; dioleostearin, 58; oleodistearin, 29; tristearin, 0.

In the second method of fractionation, the free fatty acids were extracted with dilute alkali, the neutral fat was saponified, and the unsaponifiable material was obtained by extraction. Table 9 contains the data on the fractions obtained by this procedure.

TABLE 9.—*Free fatty acid and unsaponifiable content of lard fractions*

Lard fraction	Content of free fatty acids	Content of unsaponifiable matter
	Percent	*Percent*
Filtrate (38)	0.24	0.12
Residue (39)	.23	.29

All the fractions were dissolved in tricaprylin (specimen D). The free fatty acid and unsaponifiable fractions were used in the proportions in which they occurred in lard. Three fractions were dissolved to give 10-percent solutions, by volume, in the vehicle. Fractions IV F and IV R were only slightly soluble; hence saturated solutions containing 1.8 and 1.5 percent, respectively, were employed. Benzpyrene was added to each of these solutions, and to tricaprylin alone which served as the control for the effect of the vehicle itself. The results of the experiments with these preparations are summarized in tables 10 and 11.

TABLE 10.—*Effect of fractions of lard filtrate, in tricaprylin [1] as vehicle, on the production of subcutaneous tumors in mice with 0.065 mg. of 3,4-benzpyrene*

Experiment No.	Lard filtrate		Mice [2] with tumors (+) and negative survivors (−), after various periods		
	Fraction	Concentration in tricaprylin	4 months	6 months	9 months
Average of L-21, L-24, 85, 46, and 74.	*Control average*	*Pct. 0*	*Pct. 5+*	*Pct. 32+*	*Pct. 43+*
85	Control	0	14+ 84−	58+ 38−	60+ 28−
87	I F	10	12+ 82−	40+ 40−	44+ 22−
88	II F	10	8+ 90−	32+ 52−	38+ 34−
89	III F	10	10+ 84−	40+ 52−	56+ 32−
90	IV F	1.8	0+ 94−	4+ 76−	4+ 68−
101	Free fatty acid	.24	16+ 84−	58+ 42−	66+ 28−
103	Unsaponifiable	.12	16+ 78−	44+ 46−	58+ 26−

[1] Specimen D.
[2] 50 mice, equally divided between the sexes, were used in each experiment.

The incidence of induced tumors obtained with the various fractions of lard filtrate is given in table 10. These solutions all contained 0.065 mg. of 3,4-benzpyrene per 0.2 cc. of the volume injected.

TABLE 11.—*Effect of fractions of lard residue, in tricaprylin [1] as vehicle, on the production of subcutaneous tumors in mice with 0.1 mg. of 3,4-benzyprene*

Experiment No.	Lard residue		Mice [2] with tumors (+) and negative survivors (−), after various periods		
	Fraction	Concentration in tricaprylin	4 months	6 months	9 months
Average of L-14, L-22, L-25, and 86.	*Control average*	*Pct. 0*	*Pct. 19+*	*Pct. 56+*	*Pct. 65+*
86	Control	0	28+ 166−	58+ 36−	66+ 16−
91	I R	10	26+ 172−	60+ 38−	70+ 22−
92	II R	10	25+ 166−	64+ 28−	66+ 12−
93	III R	10	38+ 160−	74+ 20−	78+ 16−
94	IV R	1.5	2+ 198−	2+ 96−	4+ 48−
104	Free fatty acid	.24	24+ 170−	60+ 32−	68+ 16−
106	Unsaponifiable	.31	30+ 168−	60+ 26−	72+ 14−

[1] Specimen D.
[2] 50 mice, equally divided between the sexes, were used in each experiment.

Fraction IV F exhibited a pronounced retarding effect on tumor genesis: tumors were produced in 9 months in only 4 percent of the mice. Fractions I F and II F gave a tumor yield of 44 and 38 percent, respectively, at 9 months as compared with a value of 43 percent for the average value of five control experiments with this dose of tricaprylin; these values are considered the same as the average value for control experiments.

Three fractions (III F, free fatty acid, and unsaponifiable) yielded higher results, which were close to those of the control group in this set of experiments. However, this particular control group (experiment 35) gave an unusually high tumor incidence as compared with the values obtained with 0.065 mg. in four other control experiments. On comparing the results given by the filtrate fractions with that given by the single control experiment in table 10, no evidence is apparent of a promoting action by any of the fractions. Although 0.065 mg. benzpyrene in lard filtrate (table 1) reproducibly gave definitely higher results than in tricaprylin, the source of this promoting effect was not clearly located in any of the filtrate fractions.

A possible explanation, which is susceptible to experimental test, is that one or more of the three fractions which gave the highest results may contain the material responsible for the promoting effect of lard filtrate, but that the enhancing influence was obscured by the abnormally high value given in this particular control experiment (85). Repetition of the experiments with the free fatty acid, unsaponifiable, and III F fractions, together with an additional control group, might provide the desired information. If the control value should be near the average value for this dose of benzpyrene, high values yielded by these lard fractions would then take on added significance.

The results obtained with the fractions of the residue lard are given in table 11. All the solutions in this set of experiments contained 0.1 mg. benzpyrene per 0.2 cc. of solution. The same specimen of tricaprylin was employed in these experiments as in those in table 10. The control group (experiment 86) produced tumors in 9 months in 66 percent of the mice; this is the same value as the average of 65 percent obtained in four such control experiments. Incidentally, the control group at 0.065 mg. (table 10, experiment 85) gave results almost as high as those in experiment 86 (table 11) in which 0.1 mg. benzpyrene was employed. This is an additional indication that the value ob-

tained in control experiment 85 was unusually high.

As in the case of lard filtrate, the lowest result was obtained with the most highly saturated fraction (IV R). Strikingly low results were consistently obtained with those fractions of lard which consisted chiefly of saturated triglycerides.

Fractions I R and II R gave results which did not differ significantly from the control. Hence they behaved as did the analogous fractions from lard filtrate.

The three fractions of the residue, the analogues of which in the filtrate had given the highest results, all yielded more tumors than did the control. However, the differences are not considered significant except possibly in the case of III R. As in the preceding set of experiments, repetition of the work is necessary to demonstrate whether any fraction in the residue regularly exhibits promoting activity. Since this set of experiments was designed to bring out the influence of retarding factors, a dose of 0.1 mg. benzpyrene was employed, for this amount of carcinogen produces tumors in about two-thirds of the mice. In repeating the work with these fractions to reveal significant promoting influences, lower dosages might be employed.

Effect of Other Saturated Triglycerides

When the pronounced difference in effect on tumor production was obtained with the filtrate and the residue of lard, it was suspected that the difference in behavior might be attributable to the difference in physical state of the vehicles for the benzpyrene. At 38°, the filtrate was a clear fluid which readily passed through filter paper. The residue was a pasty mass which was retained by the filter; it appeared to be solid fat with admixed liquid fat, for it all became liquid at a slightly higher temperature.

Tricaprylin was added to the lard residue in an amount (30 percent) sufficient to give a homogeneous, clear, liquid fat mixture. In an analogous fashion enough tristearin, a solid fat (5 percent) was added to the lard filtrate to give a pasty mass of about the consistency of the lard residue. To both preparations benzpyrene was added to give a concentration of 0.1 mg. of carcinogen per 0.2 cc. of solution at the temperature of injection. The results are summarized in table 12.

Table 12.—*Effect of physical state of lard fractions on production of subcutaneous tumors with 0.1 mg. of 3,4-benzpyrene*

Vehicle	Concentration of added triglyceride	Physical state at 38° C.	Mice [1] with tumor (+), and negative survivors (−), after various periods	
			4 months	7.5 months
			Percent	*Percent*
Lard residue		Paste	0+	8+
			100−	84−
Do	Tricaprylin, 30 percent.	Fluid	0+	0+
			100−	82−
Lard filtrate		do	88+	94+
			8−	2−
Do	Tristearin, 5 percent.	Paste	10+	10+
			88−	88−

[1] 50 mice, equally divided between the sexes, were used in each experiment.

This dose of benzpyrene in lard residue produced tumors in 8 percent of the mice in 7.5 months. Making this material liquid by addition of tricaprylin did not increase tumor production. Since 0.1 mg. benzpyrene in tricaprylin regularly produced tumors in about two-thirds of the mice (table 4), it is clear that the retarding effect of lard residue on carcinogensis was not owing to its being a semisolid paste at 38°. Apparently this material contained

some component which interfered with the action of benzpyrene.

Admixture of 5 percent tristearin to lard filtrate lowered the tumor production at 7.5 months from 94 to 10 percent. Inasmuch as the preceding experiment showed that the physical state of lard residue did not account for its retarding effect on carcinogenesis, the results obtained on addition of tristearin raised the question whether the retarding influence resided in certain of the saturated triglycerides. In support of this possibility are the data in table 8, second column, which show that the residue contained 20 times as much of the most highly saturated component (IV R) as did the filtrate (IV F). When these fractions were dissolved in the tricaprylin in amounts less than 2 percent they produced a striking reduction in the incidence of induced tumors (tables 10 and 11).

A few exploratory experiments were conducted with tristearin and tripalmitin dispersed in tricaprylin as vehicle. The results are given in table 13. In the first pair of experiments, tricaprylin (specimen E) containing 0.065 mg. benzpyrene per 0.2 cc. of solution was employed as the control. In experiment 56, tristearin was mixed with the solution in the ratio 1:19; the amount injected was slightly greater than 0.2 cc. to allow for the increase in volume. Tumors were induced after 9 months in 44 percent of the control mice, whereas the tristearin-tricaprylin mixture gave entirely negative results.

For comparison, the results with fraction IV F are also given in table 13. Here, too, a strikingly low tumor production resulted when the benzpyrene-tricaprylin solution contained only a small amount of material rich in glycerides composed of saturated fatty acids of high molecular weight.

TABLE 13.—*Effect of tristearin and tripalmitin, in tricaprylin as vehicle, on the production of subcutaneous tumors in mice with 3,4-benzpyrene*

Experiment No.	Triglyceride	Concentration in tricaprylin	Benzpyrene injected	Mice [1] with tumors (+) and negative survivors (−), after various periods		
				4 months	6 months	9 months
		Pct.	*Mg.*	*Pct.*	*Pct.*	*Pct.*
46	Control	0	0.065	4+ 88−	32+ 54−	44+ 32−
56	Tristearin	5	.065	0+ 96−	0+ 84−	0+ 84−
85	Control	0	.065	14+ 84−	58+ 38−	60+ 28−
90	IV F	1.8	.065	0+ 94−	4+ 76−	4+ 68−
86	Control	0	.1	28+ 66−	58+ 36−	66+ 16−
94	IV R	1.5	.1	2+ 98−	2+ 96−	4+ 78−
95	Tristearin	.4	.1	20+ 78−	44+ 50−	60+ 26−
96	Tripalmitin	1.0	.1	6+ 94−	40+ 58−	46+ 28−

[1] 50 mice, equally divided between the sexes, were used in each experiment.

Saturated solutions of tristearin and tripalmitin were then made in tricaprylin containing 0.1 mg. benzpyrene per 0.2 cc. of solution. A slight difference from the control was obtained with tristearin (experiment 95). A still lower tumor incidence was obtained with tripalmitin (experiment 96). For comparison the results given by fraction IV R are recapitulated in table 13. However, the retarding effect on carcinogenesis in experiments 95 and 96 was much less pronounced than in the other three experiments (56, 90, and 94).

It would appear from these few experiments that in order to obtain striking retardation of tumor genesis by benzpyrene under these conditions more than 1 percent of tripalmitin or tristearin is required. As yet there is no information available on the identity of the particular compounds

present in fractions IV F and IV R which exhibited pronounced retardation of tumor production. Since the triglycerides that occur in lard are chiefly of the mixed type, the saturated triglycerides present in these two fractions are, for the most part, presumably of the type of palmito-distearin and stearo-dipalmitin. It would be of interest to obtain data with such triglycerides and with other components which may occur in fractions IV F and IV R.

Effect of Added Substances

In parallel with the work on the reproducibility of tumor production by benzpyrene solutions in control experiments, other experiments were carried out in which various substances were added to the injected benzpyrene solution. Apart from a number of basic materials from coal tar, all the 42 materials tested were compounds that occur in the animal organism. Among the substances examined were purines and amino acids.

These substances were tested in the hope of developing some leads on promoting or retarding influences concurrently with the working out of a standard technique of known reproducibility for control groups. While no conclusions have been drawn concerning the influence of these substances on tumor genesis, the results given in exploratory experiments with some of these substances are of sufficient interest to warrant further study of their action.

In commenting on the results obtained in early experiments of this nature in which lard filtrate was employed as the vehicle, it was stated (*20, p. 226*) that the production of subcutaneous tumors in mice by a border-line dose of benzpyrene was apparently being promoted by a number of nitrogen-containing ring compounds, but that further work was required to ascertain the significance of the results of the exploratory experiments. It has since been found that while differences were often obtained between test and control groups in a single set of experiments, the variability between the results in duplicate experiments with both test and control groups, when the whole set of experiments was repeated, was such as to leave uncertain the significance of the differences.

When some of the substances were tested repeatedly, however, the incidence of induced tumors was sufficiently different from the average control value, and tumors were obtained so consistently, as to raise the question whether actual retardation occurred. Viewed in this way, not as established conclusions but rather as leads for further experimental investigation, it was found that the most pronounced reduction of tumor incidence was obtained with xanthine, nucleic acid, histidine, and nicotinic acid. Low tumor incidences resulted with regularity when each of these substances was added to the injected benzpyrene in solution both in lard filtrate and in tricaprylin. In single experiments in which the substance was incorporated into the benzpyrene solution in filtered lard, pronounced retardation was given by *d,l*-phenylalanine, tyramine, tyrosine, and hypoxanthine.

Incorporation of guanine in benzpyrene-lard solution yielded very few tumors in a single experiment; in benzpyrene-tricaprylin solution, however, the results were definitely lower than the average control value in only one of two experiments.

Among the anomalous results may be mentioned those with tryptophane and caffeine. When lard filtrate was the vehicle, tumor production in two experiments with tryptophane was about the same as in the controls. However, when tricaprylin was the vehicle, the results were lower in each of four experiments than in the controls. With caffeine in tricaprylin, there was evidence of retardation in both

of two experiments; in lard filtrate, however, the results did not differ much from those in the controls.

As data accumulated in this phase of the investigation, it became evident that the conclusions which might be drawn from an individual set of experiments might be erroneous even though 50 animals were employed in the control and in each of the test groups. Because of the variations in the results of experiments done in presumably the same fashion, the effect of substances placed at the site of injection of benzpyrene should be determined repeatedly before the evaluation of their role is considered reliable. The retarding effect of the substances mentioned earlier in this section are to be considered only as a suggestive finding in exploratory experiments and not as a conclusively established phenomenon.

TABLE 14.—*Effect of sex on the production of subcutaneous tumors in mice with 3,4-benzpyrene*

Vehicle	Experiments performed	Benzpyrene injected	Sex of mice	Number of mice	Mice with induced tumors		
	No.	*Mg.*			*No.*	*Pct.*	*Ratio* [1]
Lard filtrate	32	0.1	Male	436	269	62	1.3
			Female	483	220	46	
Do	9	.065	Male	225	191	85	1.4
			Female	225	134	60	
Tricaprylin	3	.2	Male	113	81	72	1.0
			Female	145	101	70	
Do	13	.1	Male	323	233	72	1.5
			Female	321	152	47	
Do	8	.08	Male	200	98	49	1.7
			Female	200	57	29	
Do	40	.065	Male	1,010	328	32	1.7
			Female	1,010	188	19	
Do	3	.05	Male	62	28	45	1.5
			Female	59	18	31	
Total	108		Male	2,369	1,228	52	1.4
			Female	2,443	870	36	

[1] Ratio = $\frac{\text{Percent males with tumors}}{\text{Percent females with tumors}}$.

EFFECT OF SEX

In all experiments both sexes were employed in order to determine whether they would respond differently. Table 14 summarizes the results of 108 experiments in which 2,369 male mice and 2,443 female mice were employed. In this large number of experiments the constant factors were the strain of mouse, the carcinogen and the volume injected; the dose, the vehicle, and the presence or absence of added substances in the benzpyrene solution varied from experiment to experiment. However, in each instance both male and female mice were employed; and in 84 of the individual experiments the numbers of males and of females were equal.

At every dose level from 0.1 mg. benzpyrene to 0.05 mg., more subcutaneous tumors were induced in the males than in the females. The over-all tumor incidence for each sex was calculated by dividing the total number of mice of that sex with tumors by the total number of such mice employed. The tumor incidences for male and female mice were 52 and 36 percent, respectively. In these experiments, therefore, the incidence of induced tumors was 44 percent higher in the males than in the females.

When the tabulation was restricted to the 84 experiments in each of which equal numbers of males and females were employed, the averages for tumor incidence in the two sexes were identical with over-all values given in table 14, i. e., 52 percent for the males and 36 percent for the females. The ratio of percent incidence in males to that in females was 1.4. On further restricting the calculation to the control experiments, i .e., those in which no test compounds were added to the benzpyrene-vehicle solution and in which equal numbers of animals of both sexes were employed, the same value was obtained for the ratio of tumor incidence in the two sexes. It is therefore evident that the over-all averages represent a

phenomenon obtained regularly in the various types of experiments.

The data in table 14 include the results of a variety of experiments at each dose level of benzpyrene. Data from control experiments are summarized as well as those from experiments in which substances were added to promote or retard tumor production. For this reason deductions regarding relationships between dose of carcinogen and tumor production should not be drawn from them; these relationships have been summarized in table 4 which is restricted to the results obtained in control experiments, in which only benzpyrene was contained in the tricaprylin.

Table 15 shows the ratio of the responses of the sexes when the experiments are grouped according to the level of tumor production. The first column gives the five groups according to which the experiments were classified; for this purpose the tumor incidence in each experiment was calculated by dividing the total number of induced tumors for both sexes by the total number of mice employed. The second column gives the number of experiments performed at each level of the tumor production range. Comparative data on the incidence of induced tumors in each sex are given in the other columns. For example, there were 27 experiments in each of which the combined tumor production for both sexes had a value between 0 and 20 percent. Of the 594 male mice employed, tumors were induced at the injection site in 111, an incidence of 19 percent, whereas the analogous value for the 41 tumors induced in the 596 female mice was only 7 percent. The ratio of these values was 2.7.

The values for this ratio progressively decreased as the level of tumor production increased. The lowest ratio, 1.2, was obtained in those experiments in which tumors were produced in 80 to 100 percent of the mice.

Tumor production was not invariably greater in the males. The percent incidence and the sex ratio were therefore calculated for each of the 108 experiments. In a few instances the incidence was the same for both sexes; in a few others, it was greater in the females. The variability of the ratio from experiment to experiment in any one group was greatest in the first one, i. e., the group in which the lowest yields of tumors were obtained (0–20 percent). The few experiments in which ratios less than 1 were obtained fell chiefly in this group.

TABLE 15.—*Effect of sex on the production of subcutaneous tumors in mice with 3, 4-benzpyrene in relation to the level of tumor production.*

Level of tumor production (percent)	Experiments	Sex of mice	Number of mice	Mice with induced tumors		
				No.	Pct.	Ratio[1]
0–20	27	Male	594	111	19	2.7
		Female	596	41	7	
21–40	26	Male	585	217	37	1.9
		Female	605	113	19	
41–60	23	Male	512	339	66	1.7
		Female	529	213	40	
61–80	18	Male	421	324	77	1.4
		Female	441	239	54	
81–100	14	Male	257	239	93	1.2
		Female	272	215	79	

[1] Ratio = $\frac{\text{Percent males with tumors}}{\text{Percent females with tumors}}$

The small number of experiments which yielded no significant difference between the sexes fell largely in the highest incidence range (81–100 percent). However, since sufficiently large numbers of mice of both sexes had been employed, a difference in response between male and female was obtained even in this range. The ratio (incidence in males: incidence in females) was only 1.2, but calculation of the statistical significance indicated that even this

low ratio represented a genuine difference in response between the sexes.

It was in the middle portion of the range, i. e., in those 67 experiments in which the tumor incidence was between 20 and 80 percent, that the sex difference was demonstrated most consistently in the individual experiments.

DISCUSSION

As stated in the introduction, the mechanisms involved in the genesis of tumors by substances like benzpyrene are almost entirely obscure. There is evidence from several lines of investigation that substances of biologic origin may promote or retard carcinogenesis by various agents. Various workers have reported on the effect of such materials, when incorporated into the diet or applied locally, on the genesis of tumors of the skin by coal tar, polycyclic hydrocarbons, or ultraviolet light; others have found that such substances influence the production of hepatic tumors when incorporated into the diet together with *p*-dimethylaminoazobenzene. More pertinent to the present experiments are the findings of those who studied the effect of various lipoid materials when used as vehicles for hydrocarbon carcinogens in the production of subcutaneous sarcomas. This literature was recently summarized by Dickens and Weil-Malherbe (*22*), who extended the findings of previous investigators on the inhibiting effect of mouse fat on sarcoma production with benzpyrene; their own preparation of mouse fat had been purified and freed from phospholipins. Injection of the same dose of benzpyrene in sesame oil or in arachis oil on the other hand, gave rise to tumors in a large proportion of the animals. They stated that it is not necessary for the lipoid material to be of homologous origin in order to obtain suppression of carcinogenic activity. They, too, were of the opinion that in such experiments the amount of carcinogen should not be too great, or any inhibitory tendency might be masked by its excessive activity.

Watson and Mellanby (*6*) painted the skin of mice with a petroleum-ether extract of mouse fat before tarring and obtained promotion of tumor genesis. In another experiment they included a large amount of butter fat in the diet before painting the skin with tar. This diet produced a greasy skin, and the mice had a higher incidence of induced skin tumors than those fed a control diet. Rusch, Baumann, and Kline (*23*) reported that local application of certain lipoids accelerated tumor production with ultraviolet light while linseed oil retarded it. Baumann and Rusch (*24*) also obtained an increased production of skin tumors with ultraviolet light in animals fed a high fat diet. In agreement with the finding of Watson and Mellanby, they noted that the skin of the mice on such a diet became greasy, and they raised the question whether a film of fat on the skin intensified the radiation effect. Tannenbaum (*8*) reported that a high fat diet gave an increased incidence of skin tumors induced with benzpyrene and of spontaneous mammary-gland carcinomas; the incidence of primary pulmonary tumors was unaffected while that of sarcomas induced by the subcutaneous injection of benzpyrene was unaffected or inhibited.

With another class of carcinogens, the azo dyes, a good deal of work has been done on the influence of various dietary factors on the genesis and development of hepatomas. It has been found that certain normal food constituents may affect the production of tumors of the liver. For example, White and Edwards (*25*) found that supplementing the diet with *l*-cystine increased the incidence of hepatic

tumor formation with *p*-dimethylaminoazobenzene from 60 to 96 percent.

When the present experiments were undertaken, it seemed likely that tumor production with polycyclic hydrocarbons might be found to be influenced by incorporating into the injected benzpyrene solution substances that occur biologically. It was found that when a few experiments were performed, together with a control group, interesting differences in tumor production were obtained. However, when both the test and control experiments were done repeatedly, with 50 mice in each group, it was found that in 2 given sets of experiments the differences from the control groups sometimes were consistently in the same direction, but in other instances they were in opposite directions. Furthermore, there were sometimes rather wide differences among control experiments in which the same dose of carcinogen was employed.

As was stated some years ago (*20, p. 227*), evidence had been obtained which indicated that variable results were produced with 0.1 mg. benzpyrene when dissolved in different batches of lard filtrate. The experiments summarized in table 2 confirm and extend these earlier findings.

Lard, of course, is a complex mixture of many substances, and it varies in composition from batch to batch. When synthetic preparations of tricaprylin were employed, it became clear that the characteristics of the specimens were important. Table 3 shows that consistently lower yields of tumor were given with specimen C as compared with specimen B. These preparations were the mother liquor and the crystals, respectively, obtained on crystallization of a sample of synthetic tricaprylin. For this reason the melting points of the specimens were recorded.

Table 1 shows the reproducibility obtained in two pairs of control experiments in which the same specimen of lard filtrate was employed throughout. In each case the duplicate experiment was performed at a later period. Nevertheless, excellent reproducibility of tumor incidence was obtained. From these data it would appear that sufficient basis existed for the belief that under standard conditions essentially similar results would be obtained. However, in experiments E-6 and E-10 (table 5), performed with the same dose of benzpyrene dissolved in the same batch of tricaprylin, the reproducibility was much poorer.

For these reasons, to obtain a sound basis for deductions with regard to promoting or retarding influences in experiments such as these may require multiple repetitions of both the control and test experiments. Of course the more striking the effect of a given factor on the incidence of induced tumors, the less necessary it is to repeat the experiment in triplicate or quadruplicate. In experiments 56, 90, and 94 (table 13) the tumor incidence was so low that it was obviously significant, in view of what had already been established on the range of variability in control groups at these dose levels. Only qualitative evidence of promotion or retardation was sought; no attempt was made to estimate differences of potency in quantitative terms.

For those unfamiliar with the statistical treatment of biologic data, it may be of value to point out certain precautions to be borne in mind when use is made of dose-response curves for carcinogens, such as those of Bryan and Shimkin (*11*). While these authors stated (*11, p. 831*) that "the methods can and do visualize the accuracy and the reproducibility of the experimental results," they also called attention to the fact that "they cannot be any more accurate than the facts upon which they are based." They also found

that on the basis of available data approximately 100 animals would be required to give estimates of potency accurate to $\pm$ 0.3 of a logarithmic interval or, in arithmetic terms, accurate to $\binom{+100}{-50}$ percent. This variability appears to be small when expressed in logarithmic terms but is relatively large in terms of dosage, i. e., the response to a given dose, as expected from the curve, might also be given by half or double that dose. This may also be expressed in terms of the limits of variation in the tumor incidence at any given dose. For example, the ideal tumor incidence expected for 0.0625 mg. of 1,2,5,6-dibenzanthracene (*11, table 12*) is 56 percent, but values as low as 39 or as high as 78 percent might be obtained by chance when 100 animals are used. Results in control groups accurate within this range are useful for certain purposes. However, they are of little value for the detection of modifying influences which produce in test groups changes of this magnitude in the action of the carcinogen.

In considering the limits of accuracy in tumor induction with ultraviolet radiation Blum, Grady and Kirby-Smith (*12, p. 89*) concluded, on the basis of their extensive data:

> Strictly, these findings apply only to induction of tumors by ultraviolet radiation, but it is probable that the wide variation found here is not unique in the field of cancer. If comparable latitude is found elsewhere, certain types of comparison in common use may prove unreliable.

The data reported in the present communication bear out the expectation of these authors.

By suitably increasing the number of control experiments and the total number of mice used in such control experiments, the accuracy of the dose response may be determined within narrower limits. There are a number of ways in which this may be done. In the present study the response was ascertained at 5 dose levels of benzpyrene in solution in tricaprylin. Three experiments were made with 0.05 mg. benzpyrene, 5 with 0.065 mg., 1 with 0.08 mg., 4 with 0.1 mg., and 2 with 0.2 mg. A total of 755 strain A mice was employed in these particular experiments. The mean values obtained by repeated determinations at these dose levels have far greater stability than values which might be estimated from a dose-response curve based on a small number of animals. Moreover, the data obtained in these control experiments could serve, if desired, as material for calculation of a dose-response curve of relatively high stability over the dose range studied.

It might be argued that unless a given factor exercises a striking effect on tumor genesis it is of little interest, and that it is hardly worth while to perform experiments on a large scale in order to increase the accuracy of control values if that is required to establish the significance of factors of less than overwhelming effect of carcinogenesis. However, it is conceivable that some types of so-called spontaneous tumors may arise as a consequence of the action of marginal amounts of carcinogens of biologic origin. In that event, certain factors might operate to facilitate the action of such carcinogens and others to oppose their carcinogenic action. Such speculative considerations envisage the possibility that some of these factors might be important even though their effects were less than overwhelming.

The influence of sex is a case in point. Experiments with some 4,800 strain A mice equally divided between the sexes showed that subcutaneous tumors were induced in 44 percent more males than females. In this connection the epidemi-

ologic situation in man is of interest. Many types of malignant tumors occur more frequently in men than in women. For example, leukemia, lymphosarcoma, Hodgkin's disease, gastric carcinoma, and primary pulmonary carcinoma in human beings have a higher incidence in males than in females (*26, 27*). One of the hypotheses which have been offered in explanation of the difference in sex distribution of these nepolasms is the greater exposure of men to certain postulated environmental conditions that facilitate the genesis of tumors of these types. However, the findings of the present study, in which both sexes were exposed to the same environmental influences, raise the question whether sex physiology plays a role in the higher incidence of some of these types of tumors in men.

Fieser (*16*) summarized the earlier work, particularly of Andervont, of Boyland and Warren, and of Dunning, Curtis, and Bullock, with reference to the influence of sex on the genesis of subcutaneous tumors by polycyclic hydrocarbons. He stated (*16, p. 56*):

> It may be concluded that with a large majority of the species and strains currently used in studies of induced carcinogenesis sex plays no significant role in determining the response to tumor-producing hydrocarbons.

The only apparent exception was noted in the C57 Black strain; in these mice the latent period was shorter in the males than in the females in two experiments. However, the tumor incidence in one of these two experiments was significantly lower in the males.

There may be several reasons why the sex difference, found so consistently in our experiments, was not seen in the literature summarized by Fieser. The most obvious one, however, would appear to be the relatively large doses of carcinogen employed; the lowest dose was 0.8 mg. of hydrocarbon. In most of the experiments summarized by Fieser, tumors were produced in a high proportion of the animals. In our experiments the sex difference was least noticeable when tumors were produced in more than 80 percent of the mice.

In the Japanese work summarized by Cook and Kennaway (*28, p. 539*) administration of "androstin" (a "male hormone") together with 4′-amino-2,3′-azotoluene resulted in the production of hepatomas in more male rats than in other experiments in which the azo compound was given alone or together with cholesterol; none of the females in any of the three groups showed hepatomas. These results were in agreement with the earlier ones obtained by Tomita in which a male-hormone preparation had a considerable accelerating action upon the development of hepatomas by this azo compound in male but not in female rats. The authors, Amano and Tomita, were inclined to correlate their results with the greater prevalence of cancer of the liver in men than in women in Japan. Cook and Kennaway also summarized the work of Ito who gave this same azo compound to normal and castrated female rats; castration appeared to hasten the development of hepatomas.

In the section of their review entitled "Oestrogens in Relation to Tumours," Cook and Kennaway brought together a large amount of interesting data obtained by various workers. For example, Lacassagne observed sarcomas and epitheliomas, following injection of estrogen, in strains of mice in which they were known not to have occurred spontaneously for several years, from which he concluded that the estrogenic hormones played a part in their production. For summaries of the contradictory literature on the effect of the gonads and sex hormones on carcinogenesis, the reader is referred to the reviews of Cook and Kennaway (*28*) and of Stewart (*29*). Stewart found that castra-

tion of mice of both sexes of the C3H strain, followed by subcutaneous injection of 0.8 mg. of 1,2,5,6-dibenzanthracene in lard solution, resulted in more rapid production of tumors in the castrates as compared with the intact control mice of each sex. This effect was evident up to the sixteenth week; subsequently, tumor production in the intact groups was sufficiently great so that these differences were no longer evident by the time the experiments were terminated. Stewart stated (*29, p. 1030*): "Whether the differences might have been brought out more strikingly by employing a weaker dose of the carcinogen it is impossible to state at present."

It is by now well-established that in C3H mice spontaneous hepatomas occur much more frequently in males than in females. The earlier work on the relation of sex physiology to hepatomas induced by azo compounds has been summarized in the papers mentioned previously in this discussion. More recently, Andervont and Edwards (*30, 31*) have found that the females of all strains receiving *o*-aminoazotoluene are far more susceptible than the males to induced macroscopic hepatic lesions and hepatomas. It therefore appears that the chemical mechanisms involved in the reactions of compounds which have a selective action on the liver are affected by sex physiology in a direction opposite to that observed in the effect of benzpyrene upon subcutaneous tissue. It is of interest in this connection to recall that direct application of hydrocarbon carcinogens to the liver showed this organ to be refractory to the carcinogenic action of these agents (*32*).

Although the processes involved in the successful growth of implants of transplantable tumors are different from the genesis of tumors by chemical carcinogens, it is worthy of note that Gross (*33, 34*) reported that sex plays a part in the percentage of takes on transplantation of mouse sarcoma 37. Male mice were found to be more susceptible than females to intradermal implantation of this tumor. Mice of both sexes, in which the tumors produced by this technique had regressed spontaneously, were reinoculated intradermally or intraperitoneally with the same tumor; most of the females were found to be resistant to reinoculation, whereas one-fourth of the males developed tumors.

No conclusions have as yet been drawn with regard to the effect, on tumor production, of the substances added to the benzpyrene solutions. The results of the preliminary experiments indicate, however, that it may be worth while to investigate further the effect of certain of these substances, particularly the purines.

Purines have been found (*35*) to have the property of making benzpyrene soluble in an aqueous medium. Among the purines tested were theobromine, theophylline, xanthine, and caffeine. A 3-percent aqueous solution of caffeine was found to bring benzpyrene into solution to a concentration of 20 mg. per 100 cc. In view of the results obtained in our exploratory experiments with xanthine, caffeine, and other purines, it would seem that further work with these substances might provide interesting findings.

Twort and Twort (*36*), in skin-painting experiments with dibenzanthracene and with benzpyrene, employed a number of fatty acids as vehicles, viz, oleic acid, butyric acid, and mixtures of oleic and stearic acids and of oleic and butyric acids. The results indicated that butyric acid diminished the potency of the carcinogen while stearic acid appeared to be without effect. They stated: (*36, p. 83*): "Perhaps the most interesting of the findings which have arisen from these experi-

ments is the confirmation of the sensitising effect of oleic acid." They also reported (*36, p. 85*): "Cells rendered abnormal by a few applications of benzpyrene quickly pass into the irreversibly cancerous phase when stimulated by oleic acid. Liquid paraffin, lanolin, and some other agents apparently have no such action." Berenblum (*37*) found that croton oil caused a noticeable augmentation of carcinogenesis when applied to the skin of mice in conjunction with a very dilute solution of benzpyrene in acetone; croton resin, a constituent of croton oil, exhibited a similar cocarcinogenic action to an even greater degree.

In the present work the most outstanding finding, apart from the influence of sex, was the effect of lard on tumor genesis by benzpyrene. In comparison with tricaprylin as the control vehicle, lard residue produced a striking retardation of tumor production whereas lard filtrate promoted it. Further fractionation indicated which components may be responsible for the promoting action and demonstrated that the retarding influence resides in the glyceride fraction richest in saturated fatty acids of high molecular weight.

SUMMARY

Quantitative experiments on tumor production were performed with about 5,000 strain A mice injected with marginal doses of 3,4-benzpyrene, i. e., with doses large enough to produce tumors in an appreciable percentage of the mice but below the dosage required for production of tumors in all the animals. A single injection of benzpyrene was given subcutaneously in the dose range of 0.05 to 0.2 mg. of the carcinogen. Various lipoid materials were used as vehicles for the benzpyrene. In most of the 108 experiments, 50 mice, equally divided between the sexes, were used.

Lard filtrate was the vehicle in five experiments with 0.1 mg. benzpyrene. Tumor production varied widely in these experiments. At 6 months, for example, the incidence of induced tumors ranged from 25 to 95 percent. These variations were so large as to militate against the use of this material as a vehicle in certain types of quantitative studies.

The variations were apparently a consequence of the different proportions of retarding and promoting substances in the various specimens of lard filtrate employed. Lard residue exercised a striking retardation of tumor genesis by benzpyrene. On the other hand, when lard filtrate was the vehicle, rapid tumor production in a high proportion of the animals was usually obtained. Fractionation of both the filtrate and residue of lard by various methods showed the retarding influence to reside in the glycerides which were richest in saturated fatty acids of high molecular weight.

Several other materials were tested for suitability as vehicles in quantitative experiments. The most suitable of these was found to be tricaprylin. Synthetic preparations of this triglyceride were found to vary in their effect on carcinogenesis; in some instances this appeared to be correlated with the melting points of the specimens.

Duplicate experiments, carried out at different times, sometimes gave results of satisfactory reproducibility. In other instances, however, repetition of experiments gave results which varied more than was considered desirable for certain types of comparison. Consequently, in ascertaining the dose-response relationships in the range in which tumors were produced in one-third to two-thirds of the animals, the experiments were performed repeatedly. In these standard reference experiments in which the vehicle was 0.2

cc. of tricaprylin, the following average values were obtained for tumor production in 12 months: In three experiments in which the dose of benzpyrene was 0.05 mg., the tumor incidence was 38 percent; in five experiments with a 0.065-mg. dose of benzpyrene, the tumor incidence was 45 percent; and in four experiments using 0.1-mg. doses of benzpyrene, the tumor incidence was 65 percent.

Exploratory experiments were carried out with a large number of substances of biologic origin incorporated into the benzpyrene solution prior to injection. Indications of a retarding effect on tumor production were obtained with some of these substances.

The incidence of induced subcutaneous tumors was about 50 percent higher in the male mice than in the female.

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Growth Rate and Number of Spontaneous Mammary Carcinomas and Riboflavin Concentration of Liver, Muscle, and Tumor of C3H Mice as Influenced by Dietary Riboflavin

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Any study of the relation of dietary factors to the growth of neoplastic tissues *in vivo* depends on a knowledge of the nutritive factors essential for the adequate nutrition of the host; and in an investigation of the effect of any specific dietary constituent upon the growth of tumor tissue, it is essential, therefore, to know first what effect such a factor would have on the host. From a practical standpoint, if it can be shown that a dietary constituent affects the neoplastic tissue more than the host, such a factor might possibly be useful as an adjunct in tumor therapy. When it can be shown that the omission of a single vitamin results in a specific syndrome in the experimental animal, which is entirely alleviated by the administration of the vitamin, it can be safely assumed that the specific effect of the vitamin on the growth of a tumor in the host can be studied. This was done in the experiments previously reported (*1*, *2*, *3*), when it was shown that the omission of pantothenic acid from the diet resulted in a definite syndrome in the host and in retardation of growth of the tumor, both of which were prevented by the addition of pure synthetic calcium pantothenate to the diet.

Two methods of approach to such studies of tumor growth would be applicable. The first involves a very rapid depletion of the host by providing a diet entirely free of the essential constituent under investigation but adequate in all other dietary essentials for normal growth. A rapid depletion of the host as well as the tumor would afford a deficiency condition of the animal less complicated by other deficiencies. In other words, such a procedure would be more specific for any given avitaminosis.

In the second procedure, just enough of the essential constituent is provided for maintenance of body weight of the animal. This method is intended to show whether or not tumor growth rate can be affected when the host has only sufficient of the essential factor to maintain body weight. It has been used in a study of the relation of essential amino acids to the growth of the spontaneous mammary carcinoma in mice by Voegtlin and associates (*4*, *5*), who found that diets containing just enough lysine or cystine to maintain the body weight of the host strikingly retarded the growth of this tumor. Both methods have been used in the present study.

The results of early investigations of

riboflavin deficiency were complicated by the inability of the investigators to prepare a diet adequate for growth, except for the absence of this constituent. Consequently the deficiency symptoms in experimental animals on the synthetic diets then available were usually delayed in appearing and often were not specific for a riboflavin deficiency.

It has already been established that the acute ariboflavinosis produced in mice by feeding a diet greatly deficient in riboflavin results in morphologic lesions described as atrophic and hyperkeratotic dermatosis, which is occasionally followed by a dermatitis, some myelin degeneration in the spinal cord, and a keratitis in the eye (*6*). The gross clinical picture of loss of hair, development of cracks and fissures in the skin, and exudate on the conjunctiva of depleted animals clearly indicates a considerable interference of normal metabolic processes.

The present paper gives the results of experiments on (1) the effect of riboflavin on body weight and food intake of normal young and adult mice, (2) the rate of growth of the spontaneous mammary carcinoma in C3H mice in various states of ariboflavinosis, (3) the riboflavin content and rate of depletion of liver, muscle, and tumor tissue, (4) the relation of dietary riboflavin to the number of tumors appearing, and (5) the survival of the tumor-bearing mice in various states of ariboflavinosis.

EXPERIMENTAL PROCEDURE

The Andervont substrain of C3H mice was used in these experiments. The advantages of the C3H strain of mice used in this investigation over many other strains for spontaneous-tumor studies was discussed in a previous paper (*2*). The mice were kept on Purina dog chow and had access to an unlimited supply of water. As soon as spontaneous tumors became visible, groups of animals selected at random were placed on the experimental diets and kept in individual metal metabolism cages with wire-cloth bottoms. Opal-glass ointment jars were used for food containers; round holes three-fourths of an inch in diameter were punched in the lids, and occasionally a perforated, loose-fitting disk was placed on top of the food to prevent spilling. The food consumption of each animal was recorded at weekly intervals. The maximum length and breadth of the tumors were measured weekly with metal calipers, and the tumor size was expressed as the product of these two dimensions. To conserve space and to give a clearer interpretation of the results, only average values are given; where pertinent, a statistical evaluation of the variability and significance of the data is included.

The composition of the basal diet (No. 66), deficient in riboflavin but otherwise adequate for normal growth of mice, is given in the following tablulation:

	Grams
Casein (Labco, vitamin-free)	18.0
Glucose (Cerelose)	51.3
Salt mixture No. 185a [1]	4.5
Cod-liver oil	3.0
Corn oil (Mazola)	10.0
Crisco	10.0
Ryzamin B (Burroughs-Wellcome)	2.5
Choline hydrochloride	.2
Wheat-germ oil	.5
Total	100.0

[1] Composed of 3.8 gm. McCollum's salt mixture No. 185 and 0.7 gm. $CaCO_3$. Trace elements were added to each kilogram of salt mixture in the following amounts: Cu, 20 mg.; I, 40 mg. as Cu_2I_2; Zn, 6 mg. as $ZnSO_4 \cdot 7H_2O$; Al, 0.03 mg. as $K_2Al_2(SO_4)4 \cdot 24H_2O$; Mn, 8.6 mg. as $MnSO_4 \cdot 2H_2O$; and Co, 8.1 mg. as $CoK_2(SO_4)_2 \cdot 6H_2O$.

The Ryzamin B in the basal diet is a vitamin concentrate prepared from rice polish. It is adequate as a source of the water-soluble vitamins for growth and maintenance of body weight in adult mice when supplemented with pure crystalline riboflavin. By actual analyses according to the method of Robertson and Kahler (*7*),

it was found that the riboflavin content of this diet varied from time to time from 0.4 to 0.6 micrograms of riboflavin per gram of diet. Although not completely devoid of riboflavin, it was sufficiently low to permit a rapid development of an ariboflavinosis in the mice. When 1.1 micrograms of riboflavin per gram of diet was added to the basal diet, adult mice were able to maintain their initial body weight. Riboflavin-supplemented diet No. 67 differs from the basal diet by containing 10 micrograms of added riboflavin per gram of diet.

RESULTS

The effect of the riboflavin-deficient diet on body weight of normal adult mice is illustrated in figure 1, curve A. There was a prompt and continuous loss in body weight. In the 9-week period, these animals lost about one-third of their initial weight. The similar effect on body weight in deficient tumor-bearing mice is shown in figure 2, curve B. Young mice, on the other hand, were able to increase in weight for the first week. After that, although they were unable to gain, they maintained their weight for about 5 weeks, at which time they began losing weight and soon became so deficient that they were not able to survive, as shown in figure 1, curve B.

The omission of an essential substance from a diet must be interpreted in the light of the effect on food consumption. The average daily food consumed by young and adult normal mice on a diet deficient in, or supplemented with riboflavin (fig. 1) was found to be remarkably constant for each group during the entire experimental period. When the deficient diet was supplemented with 10 micrograms of riboflavin per gram of diet, considerably more food was consumed by young mice eating this diet than was consumed by the young

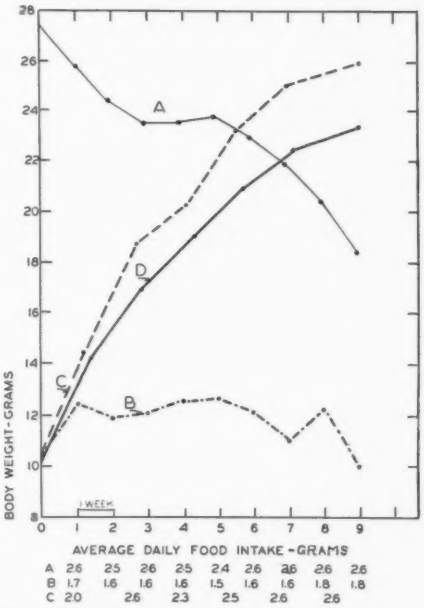

FIGURE 1.—Average body weight and average food intake of normal mice on diets deficient in and supplemented with riboflavin, and body weight of mice on stock diet. Curve A, adult deficient mice; curve B, young deficient mice; curve C, young mice on supplemented basal diet; curve D, young stock mice. Daily food intake values refer to curves A, B, and C, respectively.

mice on the unsupplemented diet, but the amount was about the same as that consumed by the adult animals which they quickly approached in size. The food intake of the young mice on the stock diet is not given because the stock diet was considerably lower in fat content than were the experimental diets, and therefore the food intake of these young mice was higher, and not strictly comparable with that of the other three groups.

In studying the effects of various amounts of riboflavin in the diet upon food consumption and body weight of tumor-bearing mice, it was found (fig. 2) that the average daily food consumed by four dif-

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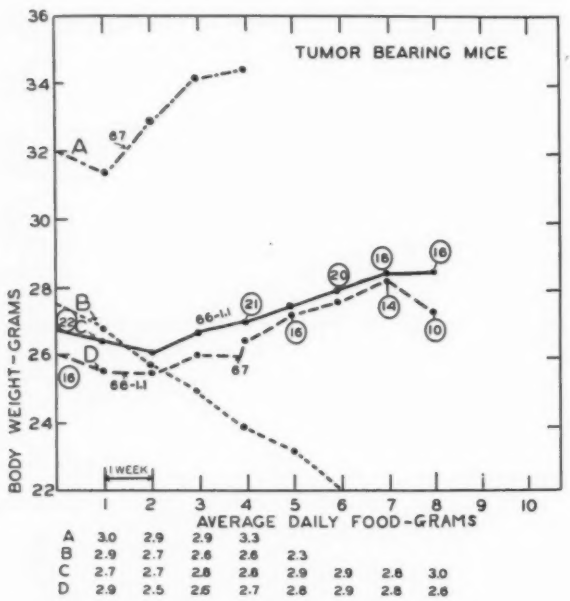

FIGURE 2.—Average body weight and food intake of tumor-bearing mice. Curve A, diet supplemented with 10 micrograms riboflavin per gram of diet; curve B, diet 66; curve C, maintenance level of riboflavin; curve D, maintenance level of riboflavin after 4 weeks, diet 67. Food intake values refer to the body-weight curves, respectively.

ferent groups was about the same. Per unit of body weight, mice on the deficient diet consumed slightly more food than did the animals receiving the highest levels of riboflavin. This increase in food consumption was because the animals on the deficient diet actually showed a considerable and continuous loss in weight, whereas the animals receiving supplements of riboflavin maintained their weight or gained slightly.

Since the normal young or adult mice consumed almost the same average daily amount of food for the entire experimental period whether on a deficient or a supplemented diet, the failure of the young animals to grow or of the adult animals to maintain their body weight on diets deficient in riboflavin was not attributable, therefore, to a failure to consume an adequate amount of food, but to a specific deficiency of riboflavin in the diet.

Female C3H mice bearing small spontaneous mammary tumors were selected at random for the study of the effect of riboflavin on the growth rate of tumors.[1] A control group of animals was studied first to obtain a measure of the growth rate of the tumor in mice on the basal diet supplemented with an excess of this vitamin. The average rate of increase in tumor size is illustrated in figure 3. The rate of growth of the tumor appears to be rapid and almost linear for the period studied (fig. 3, curve A). There were 15 tumors at the beginning of this experiment. The rate of tumor growth was next studied in mice fed the riboflavin-deficient basal diet (fig. 3, curve B). Sixty tumors were studied in this experiment. A gradually decreasing growth rate of the tumors in mice of the deficient-diet group was found as compared with that of mice in the supplemented-diet group. This decrease was more pronounced in the fourth and

[1] *The tumors used were diagnosed histologically as adenocarcinoma by Drs. S. W. Lippincott and Thelma B. Dunn.*

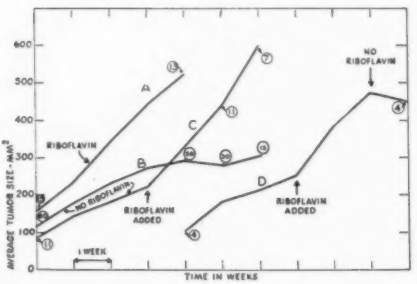

FIGURE 3.—Average rate of growth of spontaneous mammary carcinoma in mice on riboflavin-deficient and -supplemented diets. Curve A, riboflavin-supplemented; curve B, riboflavin-deficient; curve C, diet deficient for 3 weeks, then supplemented for 3 weeks; curve D, diet deficient for 3 weeks, supplemented for 2 weeks, deficient for 1 week. Circumscribed figures refer to number of tumors measured.

fifth weeks of the experiment than for any previous week. The average increase in tumor size for the supplemented-diet group during the experimental period was approximately twice the increase observed in the deficient-diet group for the same period.

The animals of the third experiment (fig. 3, curve C), which comprised 11 tumors, were placed on the deficient diet for 3 weeks, at which time they were just beginning to show gross clinical symptoms of riboflavin deficiency. The rate of tumor growth was determined during this 3-week period. The animals were then given the same amount of added riboflavin as the control group, i. e., 10 micrograms per gram of diet. The data show that the rate of tumor growth for the deficient period paralleled that of the tumors in the deficient-diet group previously mentioned. Furthermore, when riboflavin was added to the diet, the rate of growth was increased and approximated that of the control group. A fourth and smaller group (fig. 3, curve D) was given the riboflavin-deficient diet 3 weeks, then the riboflavin-supplemented diet 2 weeks, and the deficient diet again for 1 week. The tumor growth rate was greater during the supplemented-diet period but decreased when riboflavin was withheld.

In another experiment, two groups of tumor-bearing mice were placed on the basal diet to which only 1.1 micrograms of riboflavin per gram of diet was added, making the total riboflavin content approximately 1.5 micrograms per gram of diet. This is just about the level of riboflavin necessary to maintain body weight in adult mice. Only occasionally were any gross clinical symptoms of riboflavin deficiency observed in these animals after several weeks on this diet. After 4 weeks one group was placed on diet 67, which contains 10 micrograms of added riboflavin per gram of diet. The tumors of the animals receiving the maintenance level of riboflavin continued to grow

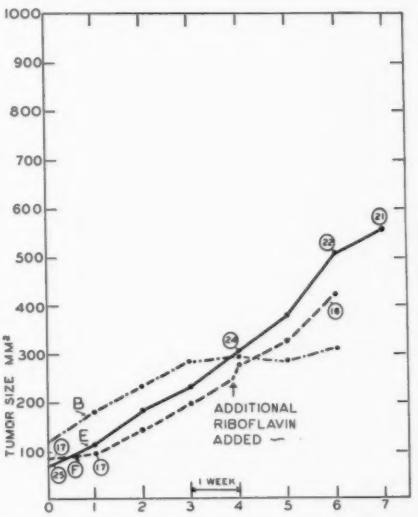

FIGURE 4.—Curve E, average rate of tumor growth on maintenance level of dietary riboflavin; curve F, same, but supplemented with additional riboflavin after 4 weeks; curve B, same as in figure 3, rate of tumor growth in mice on a riboflavin-deficient diet.

rapidly and fairly uniformly, whereas the tumors of the animals on the deficient diet showed extensive decrease in growth rate (fig. 4, curve B). The addition of more riboflavin after 4 weeks to one group (fig. 4, curve F) receiving a maintenance level of the vitamin did not result in any stimulation in tumor growth. It would appear from these data that the animal must be made extremely deficient in riboflavin before noticeable retardation of the growth of a spontaneous tumor is observed.

An attempt was made to correlate this retardation with the riboflavin content of certain representative tissues of the animal. The riboflavin content of liver, muscle, and tumor was therefore determined by its fluorescence in ultraviolet light, according to the procedure of Robertson and Kahler (7). The results are given in table 1.

TABLE 1.—*Average riboflavin concentration in mouse tissue*

Mice	Period of deficiency	Mice analyzed	Diet No.	Riboflavin per gram of dry tissue		
				Liver	Muscle	Tumor
	Weeks	*No.*		*Micrograms*	*Micrograms*	*Micrograms*
Normal	0	5	67	115.6	13.8	.....
	6	5	66	58.5	9.1	.....
Tumor-bearing	0	5	[1]Stock	112.8	16.1	[2 3] 29.9
	0	5	67	120.7	16.3	29.5
	1	5	66	100.0	12.4	[3] 29.5
	2	5	66	99.2	12.4	25.0
	3	5	66	81.5	11.6	26.5
	4	6	66	61.8	10.3	19.4
	6	7	66	64.6	11.1	16.5
Standard deviation				7.75	1.18	1.09
Standard error				4.90	.75	.69
Amount necessary for significant difference at 5-percent point				9.97	1.50	1.46

[1] Purina dog chow.
[2] Pooled sample, calculated from fresh-weight analysis.
[3] Not used in statistical calculations because the analysis was determined from a pooled sample.

Initially, the livers of normal and of tumor-bearing mice have the same riboflavin content. A gradual decrease in liver riboflavin was observable after the mice had been on the deficient diet 1 week. This decrease continued for 4 weeks, at which time about 50 percent of the original amount of riboflavin was left in the liver. No further decrease was observed after the animals had been on the deficient diet 6 weeks.

The change in muscle riboflavin was more variable than that of liver; the decrease was from about 16 micrograms per gram of dry muscle to approximately 10 to 11 after a period of 4 to 6 weeks on the riboflavin-deficient diet. Although the riboflavin of the muscle of normal mice was significantly less than that of the tumor-bearing mice both at the beginning and after 6 weeks on the deficient diet, no biologic explanation can be offered for this difference.

The riboflavin content of the tumor decreased from about 30 to 16 micrograms per gram of dry tissue, but the decrease proceeded more slowly than that of either liver or muscle and continued even after the mice had been on the deficient diet 4 weeks. Although the total decrease of the tumor riboflavin on a percentage basis was about the same as it was for muscle and liver, the rate of depletion was strikingly different.

The prompt and continuous decrease in riboflavin concentration in liver corresponded closely to the loss in body weight of the animal. On the other hand, the delayed lowering of the riboflavin in the tumor (table 1) followed closely the decreasing rate of growth of the tumor, as shown in figure 5, curves B and C. The greatest decrease occurred in both during the third to the fifth week of the vitamin deficiency.

The total size of all the tumors in the animal during the experiment were used in plotting the tumor-growth curves (fig. 5). The difference between the

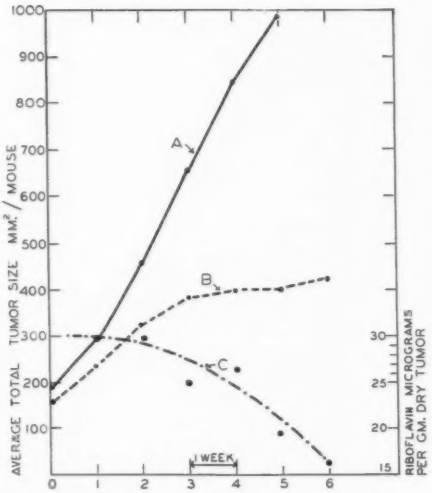

FIGURE 5.—Average total size of tumors per mouse on diets supplemented with, and deficient in riboflavin, and rate of depletion of riboflavin in the tumor in mice on a deficient diet. Curve A, tumor size of mice on complete diet; curve B, tumor size of mice on deficient diet; curve C, riboflavin content of tumor in micrograms per gram of dry weight at weekly intervals, when mice were fed a deficient diet.

basal-diet (curve B) and supplemented-diet (curve A) groups is more pronounced than in the previous tumor-growth curves (fig. 3), which were made from the averages of the individual tumors present when the animals were started on the experimental diets. This greater difference could only be ascribable to an increased number of tumors in the group receiving the riboflavin-supplemented diet. Further data bearing out this conclusion were obtained by comparing the number of spontaneous mammary adenocarcinomas in mice eating the basal diet deficient in riboflavin with the number in mice ingesting the basal diet supplemented with riboflavin during a 5-week experimental period. Initially, the animals averaged the same number of tumors per mouse, ranging from 1.2 to 1.3 per animal (fig. 6). Both groups showed an increase in the number of tumors. During the next 4 weeks the number of tumors in the riboflavin-deficient animals increased to only 1.5 per mouse, whereas during the same period the number of tumors in the animals on the supplemented diet averaged 2.7 per animal. This is a striking and significant difference. In the fifth week both groups showed a decrease in the number of tumors; the decrease was caused by the death of some animals having the highest number of tumors.

The question arises whether the increase in the number of tumors in mice on a diet containing a high level of riboflavin is an actual increase owing to some specific property of riboflavin or whether the tumors of the animals on this diet only grow more rapidly and thus become palpable and are observed before the animal succumbs. We are inclined to support the former view, namely, that riboflavin has an effect on the carcinogenesis of the spontaneous adenocarcinoma in C3H mice. In addition to the data given here, unpublished data support this hypothesis. It was found that a large group of animals receiv-

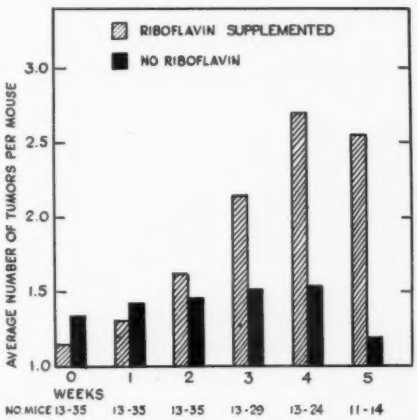

FIGURE 6.—Average number of tumors per mouse on riboflavin-supplemented diet 67 and riboflavin-deficient diet 66.

ing 10 micrograms of riboflavin per gram of diet throughout life and beginning at weaning, developed on the average 2.7 tumors per mouse after the appearance of the first tumor, exactly the same as in animals in the experiment reported herein. In another similar group receiving one-third the amount of riboflavin, the average was 2.2 tumors. The average age of these mice at the appearance of the first tumor was the same for both groups. Furthermore, the group on the maintenance level of riboflavin reported here, i. e., the group receiving one-sixth the amount of riboflavin given the high-level group, developed an average of 1.8 tumors per animal. This seems rather conclusive evidence that increasing the amounts of riboflavin up to 10 micrograms per gram of diet of the mouse results in an increasing number of spontaneous adenocarcinomas in the C3H strain.

Since the riboflavin-deficient diet decreased the number and rate of growth of the tumors, a study was made of the effect of varying concentrations of riboflavin on the survival of tumor-bearing mice.

One hundred and eleven mice were used and were placed on the experimental diets, as in preceding experiments, at the time when the tumors were first palpable, and the survival period was determined from that time until the animals succumbed (table 2).

Tumor-bearing mice on the riboflavin-deficient diet were able to survive only 40 days, or about two-thirds as long as those receiving an adequate amount of riboflavin. The mice that were placed on the deficient diet for 3 weeks before receiving the riboflavin-supplemented diet (table 2, group D), however, lived twice as long as those given only the deficient diet, and significantly longer than those given the supplemented diet for the entire experimental period. The animals of group D were able to survive longer than those of either group B or C, which received a maintenance level of riboflavin from the beginning. The biologic significance of this observation is not clear at the present time.

TABLE 2.—*Average survival of tumor-bearing mice on a basal deficient diet and on a diet supplemented with varying amounts of riboflavin*

Group of mice	Number of mice	Riboflavin supplement per gram of diet		Mean survival	Difference		
		Initial	Additional		Groups	Days	Probability
		Micrograms	*Micrograms*	*Days*			
A	46	0	.....	39.9	E–A	21.9	<0.01
B	22	1.1	.....	68.9	B–A	29.0	<.01
C	16	1.1	[1] 10	68.3	.....	.....	.....
D	12	0	[2] 10	76.1	D–A	36.2	<.01
E	15	10.0	.....	61.8	D–E	14.3	<.02

[1] Added at 4 weeks.
[2] Added at 3 weeks.
Standard deviation, 15.0.

Thus far we have found that a deficiency of riboflavin retarded the rate of growth of the mammary adenocarcinoma and decreased the survival period of the tumor-bearing animal. To answer the question, what effect has tumor size on survival time, a statistical analysis was made of the various groups fed different amounts of riboflavin. The results of these calculations are expressed (fig. 7) by the regression curves of survival in days over tumor size. The curves for each of the five groups indicate no significant deviation from zero correlation between the maximum tumor size and survival of the mice on any of the riboflavin-deficient or riboflavin-supplemented diets in this series of experiments. It would seem justifiable, therefore, to conclude that the size of the spontaneous tumors once they become palpable does not affect the length of time the animal will live, either on a riboflavin-deficient diet, a maintenance diet, or one supplemented with an abundant amount of riboflavin.

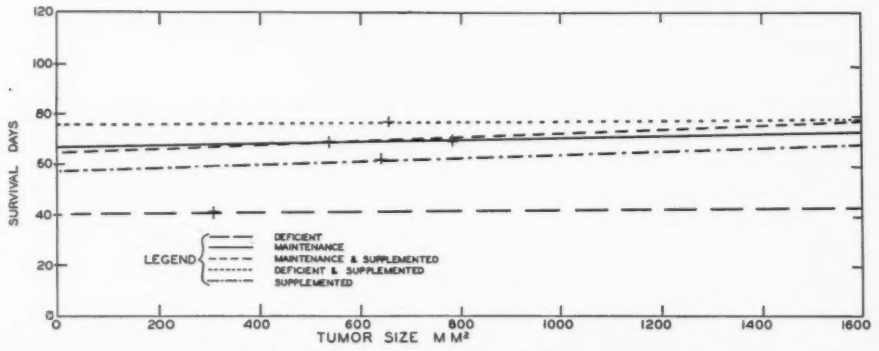

FIGURE 7.—Regression[2] curve of survival in days of tumor-bearing mice on total size of tumor expressed as square millimeters at the various levels of dietary riboflavin indicated.

DISCUSSION

The results of the present investigation permit us to draw certain conclusions regarding the relation of riboflavin to the growth of normal and neoplastic tissues. Riboflavin must be an essential constituent of both types of tissue, for neither growth nor maintenance will long continue when the riboflavin becomes deficient. With a loss of even 20 percent of the riboflavin in liver or muscle, there is at the same time a considerable decrease in body weight of adult mice. In tumors this reduction in riboflavin concentration roughly coincides with a significant slowing down of the growth rate. It seems improbable, therefore, that any of these tissues contain riboflavin much in excess of normal metabolic requirements.

Although all tissues are dependent upon an external source of the vitamin for maintenance of an adequate concentration, all are not equally sensitive to its absence from the diet. Such a difference is noted between normal and cancerous tissue. A dietary deprivation of riboflavin does not noticeably affect tumor growth or concentration until after the third week, whereas body weight and riboflavin concentration in normal adult tissue fall immediately. Since this difference in rate of depletion favors the neoplasm, any attempt to control its growth by the production of an ariboflavinosis would debilitate the host before the tumor was affected.

It was found that both the young and the adult mice deficient in riboflavin consumed almost as much food during the terminal stages of deficiency as they did at the beginning of the riboflavin-deficient regimen. Since riboflavin deficiency does not lead to a decrease in food consumption, the question is, What causes this loss in weight?

If the average daily food consumption per unit of body weight is calculated from the data in figure 1, a measure of efficiency of food utilization can be obtained. This shows a definite trend to a lowered efficiency of food utilization in the deficient animals toward the terminal stages, which was not evident in the group receiving the riboflavin supplement. Our experiments were not planned to show specifically this effect on food utilization. The effect of riboflavin on food utilization in the rat was investigated by Sure (*8*). He reported experiments in which two groups of rats

[2] *The word "regression" is used in its statistical sense.*

were fed the same amount of a basal diet, one group receiving a supplement of 20 micrograms of riboflavin daily. The group on the supplemented diet gained on the average 10 times more than did the group on the deficient diet in 125 days. The decreased deficiency of food utilization which Sure observed was not associated with any marked anorexia in the animals on the deficient diet in the terminal stages of ariboflavinosis.

The fact that we have observed a tendency toward a lowered efficiency of food utilization and a decrease in growth rate of the spontaneous mammary tumor suggest that these two functions are intimately related to the essential physiologic role of riboflavin in tissue metabolism and probably more specifically as an integral portion of one or more of the many flavoproteins concerned in biologic oxidations. The activity of some of these enzyme systems has been shown to be lower in various tissues of rats on a riboflavin-deficient diet (*8–11*).

Our observation that dietary riboflavin deficiency definitely retarded the growth of spontaneous tumors led us to study in a small series of rats[3] the effect of a pre-existing dietary deficiency of riboflavin upon the growth of transplants of hepatoma 31 (*12*). This transplanted tumor grew much more rapidly in animals on a diet high in riboflavin than in those depleted in riboflavin when fed the deficient diet beginning 2 weeks before the transplantation was made. It would appear, therefore, that riboflavin deficiency may also definitely retard the rate of growth of other types of tumors.

The further observation that riboflavin apparently stimulates the induction of additional mammary tumors at first seems in disagreement with the report of Kensler, Sugiura, Young, Halter, and Rhoads (*13*) on the retarding effect of massive amounts of riboflavin on the production of tumors in the livers of rats fed *p*-dimethylaminoazobenzene. One should keep in mind the probability that tumor growth and tumor induction are two distinct phenomena. In the case of tumor induction with the dye, it may be suggested that riboflavin acts either to preserve the normal tissues (*14*), which detoxify the dye, or that possibly the presence of large amounts of riboflavin aids directly in the detoxification process which must be going on continuously in the livers of the animals consuming the dye. In this connection, Kensler, Sugiura, and Rhoads (*15*) reported a decreased riboflavin concentration in the liver of rats fed *p*-dimethylaminoazobenzene, even before the development of the primary hepatic tumor. There are insufficient data at present to explain the role of riboflavin in the carcinogenic process, and it seems premature, therefore, to generalize on the effect of riboflavin on carcinogenesis.

[3] *Unpublished data.*

SUMMARY

The body-weight changes of both young and adult normal mice on a riboflavin-deficient diet were investigated. The rate of growth of the spontaneous mammary carcinoma in 111 C3H mice was studied at 3 levels of riboflavin in the diet: (1) Deficient; (2) maintenance; and (3) abundantly supplemented. The effect of riboflavin on the number of tumors was noted, and extensive analyses were made of the riboflavin content of tumor, liver, and muscle of both normal and tumor-bearing adult mice.

Riboflavin is essential for growth of normal young mice and for maintenance of body weight of adult normal and tumor-bearing mice. The food intake of the animals was not greatly influenced by a deficiency of riboflavin.

The growth rate of spontaneous tumors was decreased in the animals on the riboflavin-deficient diet. No appreciable difference in growth rate of the spontaneous mammary carcinoma was observed between a maintenance level of riboflavin and a riboflavin-supplemented diet. Apparently it is necessary to make the animal severely deficient in riboflavin before the growth of the tumor is affected. The most extensive effect on the reduction in tumor growth occurred in animals after 3 weeks on the deficient diet and roughly coincided with the riboflavin depletion of the tumor.

There was a marked increase in the number of spontaneous tumors in the tumor-bearing animals on feeding them a high level of riboflavin.

There was no correlation between the total size of the tumor and the length of survival of the animal on any of the different levels of riboflavin. A significant decrease was observed in survival of tumor-bearing, riboflavin-deficient mice as compared with mice receiving riboflavin. The survival period was increased by placing the tumor-bearing animals on a riboflavin-deficient diet for 3 weeks and then supplementing the diet with riboflavin.

The riboflavin content of the liver of both normal adult and tumor-bearing mice decreased about 50 percent on the deficient diet in 4 weeks, but there was not much further decrease after 6 weeks on the deficient diet. The riboflavin content of muscle decreased about a third during the same period, while that of the tumor decreased nearly 50 percent, but not so soon as in the liver and muscle.

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Degradation of Cystine by Normal Liver But Not by Transplanted Hepatomas

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It has long been known from feeding experiments with animals that the metabolism of cystine involves the breakdown of the molecule into inorganic sulfate and urea (*1*). The intermediate three-carbon acid, pyruvic acid, produces glycogen (*2*). As in the case of the degradation of other amino acids, the site for the conversion of cystine has been believed to be the liver. The first conclusive evidence for this assumption has been the work of Fromageot, Wookey, and Chaix (*3*) who showed that cysteine is degraded by the liver to yield hydrogen sulfide. These authors ascribed the action to an enzyme which they named "desulfurase." The enzyme occurs almost exclusively in the liver although traces appear to be present in the kidney and pancreas. The activity varies considerably in the livers of different species of animals (*4*). Smythe (*5*) made an intensive study of the utilization of cystine and of cysteine by liver tissue and observed that these substances were equally degraded under aerobic or anaerobic conditions. In either case sulfur, hydrogen sulfide, ammonia, and pyruvic acid were produced.

White and Edwards (*6*) have described the relation of dietary cystine to the induction of hepatic tumors in rats fed *p*-dimethylaminoazobenzene. On a low cystine diet the latent period of tumor formation is increased; on a high cystine diet tumors appear earlier. As the liver becomes neoplastic what happens to its capacity to utilize the ingested cystine? As a partial answer to this question, results were obtained on incubation mixtures of extracts of transplanted hepatomas with cystine, cysteine, and related compounds.

EXPERIMENTAL PROCEDURE

The tissues included normal rat and mouse livers, transplanted hepatoma 31 in Osborne-Mendel rats, and transplanted hepatomas E in C3H mice (*8*), 1 in I mice (*8*), and 587 in A mice (*9*). The tissues were removed immediately after the death of the animals (generally by decapitation), ground in a mortar with sand, and taken up in twice the weight of distilled water. After intermittent stirring of the mixtures for 1 hour, they were centrifuged, and the supernatant fluid was used immediately for the determination of enzymatic activity. The total N per cubic centimeter of the liver extracts was 5–7 mg., and that of the hepatoma extracts 3–4 mg. Two cubic centimeters of the extract was mixed with 1 cc. of M 5 phosphate buffer at pH 6.9. To this mixture was added 1 cc. of each of the amino acid solutions used, and the final volume brought to 5.0 cc. with distilled water. The stock solutions of the *l*-amino acids were 0.025 M, those of the *dl*-acids 0.05 M.[1] The mixtures containing the sulfur amino acids were incubated for 2 hours at 37°C. under aerobic and under

[1] *Solutions of cystine were prepared by dissolving the amino acid in an equivalent amount of normal NaOH, followed by neutralization with HCl. The appropriate volumes of such solutions were rapidly pipetted into the digestion flasks before crystallization of the cystine began. Fresh solutions were prepared before each set of determinations.*

anaerobic conditions. The pH of 6.9 at which the determinations were performed was close to the optimum value (*5*). After the end of the digestion period, acetic acid was added to bring the pH of each of the mixtures to about 5, and the digestion tubes were placed in boiling water for 10 minutes. The mixtures were then made alkaline, and the ammonia evolved was trapped in sulfuric acid and estimated by nesslerization. Blanks of the tissue extracts were run simultaneously and the values subtracted from those obtained in the amino acid digests.[2] The amino acids used were purified products. The peptides have been described (*10*, *11*). The results are given in table 1.[3]

TABLE 1.—*Ammonia N produced by the action of tissue extracts on amino acids*[1]

Substrates	Ammonia N produced with extracts of—					
	Normal rat liver	Rat hepatoma 31	Normal mouse liver	Mouse hepatoma—		
				E	587	1
	Moles x 10^{6}	*Moles* x 10^{6}	*Moles* x 10^{6}	*Moles* x 10^{6}	*Moles* x 10^{6}	*Moles* x 10^{6}
l-Cystine	9.0	0	7.9	0	0	0
dl-Cystine	9.0	0	7.8	0	0	0
dl-Methionine	.4	0	0	0	0	0
l-Cystine + *dl*-methionine	9.7	0	7.9	0	0	0
l-Cystine + *dl*-alanine	9.8	0	7.4	0	0	0
dl-Cystine + *dl*-methionine	9.2	0	6.9	0	0	0
dl-Cystine + *dl*-alanine	9.5	0	8.1	0	0	0
dl-Methionine + *dl*-alanine [2]	1.2	0	0	0	0	0
dl-Alanine [2]	1.0	0	0	0	0	0
l-Cystine + pyruvate [3]	9.3	0	.....	.....	.....	.....
l-Cysteine	3.9	0	3.0	0	0	0
Diglycyl-*l*-cystine	6.4	0	.....	.....	.....	.....
l-Cystinyldiglycine	8.0	0	.....	.....	.....	.....

[1] Figures for the sulfur-containing compounds were obtained under either aerobic or anaerobic conditions. Molarity of the cystine is calculated on the basis of 120, which is that of the half molecule.
[2] Only under aerobic conditions.
[3] The pyruvate was 0.025 M.

[2] *The ammonia in the crude extracts of hepatomas was invariably about double that in the extracts of normal liver. The ammonia N in the latter was 0.5–0.7 percent of the total N; in the former ammonia N about 1.0–1.7 percent of the total N.*

[3] *A few experiments in which the livers of the hepatoma-bearing rats were used revealed no difference in the enzymatic activity of these livers and of the livers of normal animals.*

DISCUSSION

Table 1 reveals that under conditions whereby cystine was readily broken down by extracts of normal liver, extracts of the hepatomas were completely without effect. Whether the digestions were conducted under aerobic or anaerobic conditions, substantially the same results were obtained. The presence of hydrogen sulfide was clearly detectable by its odor in the normal liver digests containing cystine, cysteine, and the cystinyl peptides, but no trace of the gas was evident in the hepatoma digests. These compounds were nearly quantitatively accounted for by the intensity of the nitroprusside reaction after KCN treatment in the hepatoma digests after dilution of the digests. The hepatomas apparently lost the capacity of the normal liver cell to degrade cystine or cysteine.

The enzyme complex in normal mammalian liver which degrades cystine is powerful. It is much more active than *d*-amino acid oxidase. As a matter of fact, it is entirely different from the latter enzyme system since it functions as well anaerobically as aerobically, and in contrast with *d*-amino acid oxidase (*12*) it is observable in mouse liver tissue. Since it is active in tissue extracts as well as in tissue slices, it is also different from *l*-amino acid oxidase (*13*). The addition of racemic amino acids to digests containing cystine apparently produces no effect, i. e., there is no competitive degradation. Cystine is apparently degraded at a greater rate than cysteine. The peptides of cystine are broken down at a rate little different from that of the equivalent amount of cystine alone. In view of the relatively weak dipeptidase activity of liver tissue (*14*), it is hard to escape the conclusion that the cystine peptides are degraded as such. The liver apparently utilizes cystine by a

system entirely different from that whereby other amino acids are broken down. The split products of the cystine, insofar as the ammonia and pyruvic acid are concerned, follow familiar paths, for the former is detoxified in the intact animal to form urea and the latter gives rise to extra glucose (*2*). The hydrogen sulfide presumably is converted into inorganic sulfate and is excreted as such. The conversion takes place in the liver, and its mechanism has been studied by Medes and Floyd (*15*).

The cystine ingested must encounter this enzyme complex when it reaches the liver. In a sense, this complex may be looked upon as a barrier to the general utilization of the dietary cystine by other body tissues. With this thought in mind, an explanation, admittedly oversimplified and certainly speculative, is advanced for the results of White and Edwards (*6*) in retarding the appearance of hepatomas in animals on a low cystine diet. When low quantities of cystine are ingested, it is possible that much, if not all, of the cystine is degraded by the liver and little escapes to become available for the growth of the tissue proteins. On the high cystine diets enough of the amino acid escapes the enzymatic barrier to become available for tissue growth. Animals on the low cystine diets are smaller than those on adequate diets, but the careful regulation of the composition of the inadequate diet, as White and Edwards have shown, is sufficient to maintain the animals in a healthy state. The appearance of the hepatoma is delayed under these conditions until sufficient cystine has passed the enzymatic barrier. The change of the liver cell into the hepatoma causes more cystine to be available for growth purposes, inasmuch as the hepatoma tissue does not degrade the amino acid; the more tumor cells and less liver cells there are, the better are the chances for cystine to participate in the hepatoma growth. In this hypothetical sense, the growth of the hepatoma favors its further growth.

No account has been taken, in this discussion, of the role of the carcinogen or of the many other changes in the enzyme systems of the liver cell when it becomes neoplastic, yet these factors must also play some part in the growth of the neoplasm. Nevertheless, the studies of White and his collaborators (*6*, *16*, *17*) appear to indicate that cystine occupies a unique position in the induction of neoplasms of different origin, and it may be advisable at the present time to emphasize the factors that influence the utilization of this particular amino acid in the animal organism.

SUMMARY

Cystine, cysteine, peptides of cystine, and mixtures of cystine with other amino acids were incubated with extracts of normal liver and of transplanted rat and mouse hepatomas. Cystine and related compounds were rapidly degraded by the normal livers of rats and of mice but were not at all attacked by extracts of the hepatomas.

The enzyme complex in the liver responsible for the degradation of cystine and its possible role in tumor induction under certain dietary conditions are discussed.

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Hydrogen-Ion Concentration of Normal Liver and Hepatic Tumors

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Liver and hepatic tumor have been considered the most suitable pair of homologous tissues available for studies of the changes which a normal tissue undergoes upon becoming cancerous. In order to elucidate the metabolic changes, such as decreased respiration and increased glycolysis, which occur, several workers have compared the concentrations of various enzymes in these two tissues (*1*, *2*, *3*, *4*). Since the activity of many enzyme systems is dependent on the hydrogen-ion concentration, any attempt to synthesize the results of these assays into an over-all picture must take this factor into consideration. It was, therefore, considered advisable to extend previous measurements of the pH of tumors to include hepatomas and normal liver. Voegtlin, Fitch, Kahler, Johnson, and Thompson (*5*), using the capillary glass electrode *in vivo*, found several sarcomas and carcinomas to have a pH of about 7.0. These tumors became more acid shortly after intraperitoneal or subcutaneous injection of *d*-glucose, mannose, xylose, fructose, or maltose, whereas galactose, lactose, arabinose, and sucrose were without effect.

The present paper describes a modification of the method for the determination of the hydrogen-ion concentration in tissues *in vivo* and the results obtained by its application to normal liver and hepatic tumors.

EXPERIMENTAL PROCEDURE

The construction of the capillary glass electrodes used in previous studies (*6*) was slightly modified as follows: A medium hard glass tube about 20 cm. by 7 mm. is constricted at one end to form a thick-walled capillary;[1] to this is sealed a globule of Corning 015 glass which is then pulled out to a fine, thin-walled capillary, cut off 10–15 mm. from the hard glass, and sealed at the tip. The capillary of 015 glass is used as the actual pH measuring surface. These electrodes are stable for at least several months and are in this respect superior to the old type electrodes made entirely of 015 glass. The electrodes are filled with phosphate buffer saturated with KCl, and all traces of air in the capillary are removed by evacuation. The electrodes are then soaked overnight in water and calibrated with two standard buffers at 37° C. before each day's use.

The potential measuring system was essentially that previously described and

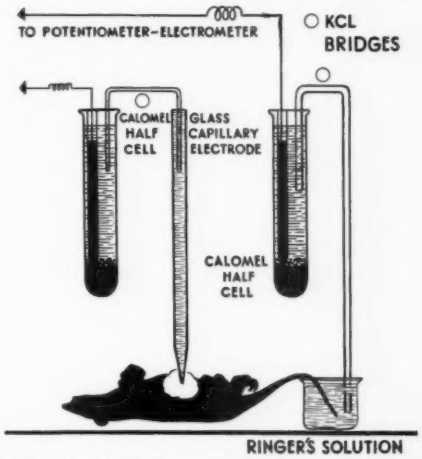

FIGURE 1.—Setup for measuring tumor pH *in vivo*.

[1] *Five-milliliter transfer pipettes manufactured by the Ace Glass Co. proved very suitable.*

consisted of KCl-agar bridges, calomel half cells, Leeds & Northrup type K potentiometer, quartz switch, and Compton electrometer. Contact with the animal was made by immersing the clipped tail in a beaker of Ringer's solution. Figure 1 illustrates the electrode and the setup used. Adequate electrical shielding was maintained by placing the whole system, including the animal, in a large, grounded cage made of fine-mesh wire.

Animals with tumors sufficiently large to accommodate the glass electrode were fasted for about 20 hours previous to making a determination. One milliliter of a 10-percent solution of ethyl urethane was injected intraperitoneally for each 100 gm. of body weight, and the anesthetized animals were fastened to an insulated board. During the experiment further urethane was administered only in minimal amounts necessary to prevent too much movement. The amount of anesthetic required to maintain a light but adequate narcosis varied considerably.

Variations in technique were necessary in order to measure properly the pH in different tissues. The electrode was inserted in the hepatoma through a small, non-bleeding, cutaneous incision. If the tumor capsule presented too much resistance to the passage of the electrode, it was punctured with a small needle. Whenever possible, the electrode was placed peripherally to avoid contact with any central necrosis. However, no consistent differences were found when the electrode passed through such necrotic areas. In the case of primary hepatic tumors [2] rats were selected which had a large palpable tumor on a major frontal lobe. The electrode was inserted through a small incision in the peritoneum. After completion of an experiment the tumors were always examined to ascertain whether the electrode had been completely embedded in cancerous tissue. In order to insert the electrode in normal liver, it was necessary to make a small incision in the peritoneal cavity just beneath the xyphoid. The left major lobe was exposed by the application of lateral pressure, then packed with saline-moistened gauze. The electrode was inserted so that it punctured the anterior surface at the edge and passed through the center toward the hilus.

After the insertion of the electrode, the animal was placed in the shielded cage, the end of the tail was clipped and dipped into Ringer's solution, and the bridges were put in place. The elapsed time between insertion of the electrode and the first determination was about 5 minutes. For a short time thereafter the electrode potential was unsteady, probably because of a slight trauma produced during insertion of the electrode.

The tumors and livers were excised from the living anesthetized animal shortly after removal of the glass electrode. Two hundred to five hundred milligrams of tissue sliced to a thickness of about 3 mm. was immediately dropped into 10 ml. of normal sulfuric acid containing about 250 mg. of sodium fluoride. The material was extracted several times by grinding in a mortar, and the combined extracts were made up to 100 ml. Lactic acid was determined on a small aliquot by the method of Barker and Summerson (*8*). Duplicates on the same extract checked within ± 2 percent, and duplicates on the same tissue within ± 5 percent. However, as may be seen from the data, the variation between identical tissues in similarly treated animals was much greater.

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[2] *These tumors, induced by feeding Osborne-Mendel rats* p-*dimethylaminoazobenzene, were obtained from Dr. Julius White and were diagnosed as hepatoma type II* (7).

RESULTS

Hepatomas in Rats

The hydrogen-ion concentration was first measured in a subcutaneously transplanted hepatic tumor, hepatoma 31 (*9*). An unsteadiness in the electrode potential, which appeared immediately after insertion, subsided within 15 minutes, after which a relatively constant value was maintained indefinitely. The average pH of tumors in 10 fasted rats was 6.99 (6.81 to 7.10).

When the potential had remained steady for about 30 minutes, 600 mg. of glucose per 100 gm. of body weight was injected intraperitoneally as a 20-percent solution. Within 10 minutes the pH started to fall, and a maximum acidity was reached 2 to 3 hours after glucose administration. Tumors of nine rats treated in this manner had an average pH of 6.42, varying only between 6.40 and 6.45. Typical data are illustrated in figure 2. The administration of a second, equally large dose of glucose at this time caused no appreciable increase in acidity. The pH remained approximately constant for several hours.

The inability to lower the pH below 6.4 by injection of excess glucose and the small variability observed in this value suggest that pH 6.4 is the limit of maximum acidity, i. e., glycolysis of hepatoma 31 *in vivo* cannot force the pH below 6.4. This may be attributable to inhibition of glycolysis by hydrogen ions, to an increased rate of diffusion of acid away from the tumor, or to a combination of the two factors. Warburg, Posener, and Negelein (*10*) showed that the glycolysis of Flexner-Jobling carcinoma slices *in vitro* decreases with a decrease in pH and is probably inhibited completely below pH 6.0. Rona and Wilenko (*11*) observed no glycolysis in blood below pH 6.4. It seems probable, therefore, that glycolysis in hepatoma 31 is also inhibited by a lowered hydrogen-ion concentration, and that the tumor is

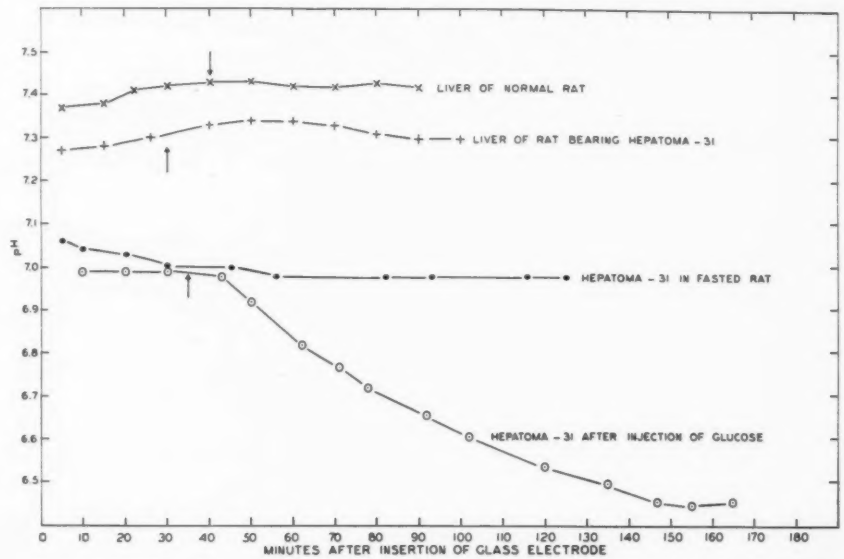
LIVER OF NORMAL RAT
LIVER OF RAT BEARING HEPATOMA - 31
HEPATOMA - 31 IN FASTED RAT
HEPATOMA - 31 AFTER INJECTION OF GLUCOSE
pH
MINUTES AFTER INSERTION OF GLASS ELECTRODE

Figure 2.—Effect of glucose administration upon the pH of normal liver and hepatic tumor.

thereby prevented from becoming excessively acid.

One purpose of this study was to determine the normal hydrogen-ion concentration of the tumor as a guide to enzymatic analysis. Since neither the fasted rat nor the one injected with a large amount of glucose is living under normal dietary conditions, the pH of tumors in rats eating dog chow *ad libitum* was determined. The pH of tumors in four rats averaged 7.02 (6.99 to 7.04) which was the same as the pH value obtained on fasted rats. Since hepatoma 31 contains only traces of glycogen (*9*), it is probable that the blood sugar must be elevated before tumor acidity can increase.

The studies on primary hepatomas in the rat did not yield such consistent results as those on hepatoma 31. The initial pH upon insertion of the electrode varied considerably, and the effect of intraperitoneal injection of glucose was much less in these tumors than in the transplanted hepatomas. The average pH in the primary hepatic tumor of six fasted rats was 7.02 (6.72 to 7.22) and fell to only 6.73 (6.35 to 6.99) 2 hours after the injection of glucose.

It was considered possible that the location of these tumors might be responsible for the effects noted, i. e., the surrounding liver might either prevent excess glucose from reaching the tumor or it might utilize acidic products of metabolism so rapidly that no excess could accumulate. An attempt was made to examine this possibility by transplanting hepatoma 31 into liver. In four of eight animals inoculated intrahepatically by Dr. Edwards, of this Institute, the tumor started to grow, but in no case was the tumor large enough to permit insertion of the glass electrode. The largest tumor had a diameter of about 15 mm. 6 months after transplantation.

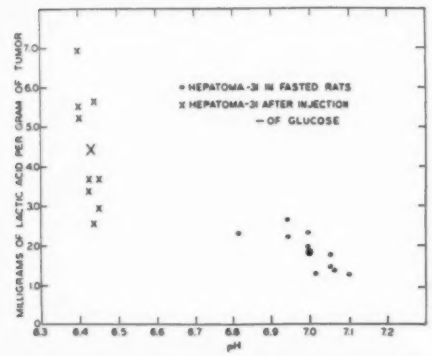

FIGURE 3.—Lactic-acid concentration and pH of hepatoma 31 before and after the injection of glucose. Large symbols indicate average values.

Glycolysis is probably the predominant metabolic process in tumors, in which case the increased acidity after glucose injection would probably be ascribable to the production of lactic acid. The average lactic-acid concentration in hepatomas in 11 fasted rats was 1.87 mg. per gram of fresh tumor (1.28 to 2.65). In 9 animals which had received glucose 3 hours previously, the lactic-acid concentration averaged 4.41 mg. per gram of fresh tumor (2.58 to 6.95). The lactic-acid concentration and corresponding pH are shown in figure 3. Despite the large variations observed, the average lactic-acid concentration in the tumors of rats to which glucose was administered was significantly higher than that found in fasted animals. The increase corresponded approximately to the increase in hydrogen-ion concentration from pH 6.99 to pH 6.43. However, within either group, fasted or glucose-injected, no correlation between hydrogen-ion concentration and lactic-acid concentration was observed. This finding is similar to those on Jensen rat sarcoma previously reported (*5*).

NORMAL LIVER

The behavior of the electrode potential in normal liver was strikingly different

from that in hepatoma 31. Even under the most careful conditions the potential was not so stable as that of the hepatoma. These drifts seemed to be real shifts in pH, for upon interchange of electrodes the pH obtained was approximately the same as the last reading obtained with the first electrode, and any drift observed in the first was continued in the second. Possibly the liver represents a more poorly buffered system than does the hepatoma.

It is obvious that only those potentials that remained reasonably constant are acceptable for evaluation. Data typical of those used are illustrated in figure 2. The pH of the livers of 12 fasted normal rats averaged 7.39 (7.18 to 7.51); the pH of the livers of 9 fasted hepatoma-bearing rats averaged 7.30. The difference in hydrogen-ion concentration in the livers of normal and tumor-bearing animals cannot be considered significant, for such a difference could occur 8 percent of the time by chance alone. The intraperitoneal injection of glucose did not change the hydrogen-ion concentration in the liver of either normal or tumor-bearing rats.

The significance of the concentration of lactic acid in liver is difficult to interpret because of the large variations which occurred. Any of several factors may be responsible for this variation, e. g., the actual degree of fasting achieved, the amount of anesthetic used, the intensity and duration of struggling while anesthetized, and the amount of cellular damage caused by the electrode. No significant differences were found in the lactic-acid concentration of fasted and glucose-injected rats, or between normal and hepatoma-bearing rats. The lactic-acid concentration in the livers of 20 rats averaged 0.82 mg. per gram (0.40 to 1.48).

Hepatomas in Mice

Hepatomas in mice present an interesting contrast with rat hepatoma 31. The cells show more histologic resemblance to the parent liver cell, and the mouse tumors are more highly differentiated than hepatoma 31 (*12*). Some of the biochemical characteristics are also more like those of liver than are the characteristics of the rat hepatoma (*9*, *12*, *13*).[3] One might well expect these differences to result in metabolic changes which would be reflected in the pH response to injected metabolite.

Hepatomas in mice available for analysis were the transplanted spontaneous and carbon tetrachloride-induced tumor in strain A mice (*15*) and the transplanted spontaneous hepatoma in C3H mice (*12*). Histologically, these tumors were indistinguishable, and it was considered justifiable to treat them as a single group. Because of technical difficulties, mouse liver could not be used as a control. However, it seems reasonable to assume that the hydrogen-ion concentration of rat liver and mouse liver would be about the same.

The behavior of the glass electrode after insertion into mouse hepatomas resembled that in normal rat liver. The average initial pH of five tumors, in which the potential remained steady, was 6.74 (6.54 to 6.93), which is considerably lower than that of normal rat liver and even somewhat more acid than that of rat hepatoma 31. The wide variation observed is, however, more characteristic of rat liver than of rat hepatoma. Upon injection of glucose, the pH of the mouse tumor falls, reaching an

[3] *Since completion of this work, Dickens and Weil-Malherbe* (14) *have reported a comparative study of the metabolism of mouse and rat hepatomas. They found that mouse hepatoma in many respects resembles normal mouse liver, whereas rat hepatoma resembles other malignant tissues.*

average low of 6.22, a behavior that is charactertistic of neoplasms.

The lactic-acid concentration in hepatomas of fasted mice was lower and did not rise as high upon glucose injection as that of hepatoma 31. The average concentration in tumors in fasted mice was 1.49, and in glucose-injected mice 1.93. This difference is not significant and certainly not large enough to account for the change in hydrogen-ion concentration.

The mouse hepatoma, like liver, normally contains glycogen. Staining techniques [4] showed further that this hepatoma was able to resynthesize glycogen at about the same rate as liver. Fasted mice showed no evidence of glucose in either liver or tumor, whereas both tissues in mice that had been injected with glucose were full of glycogen 2 to 3 hours later. This ability to utilize and synthesize glycogen further complicates the metabolic picture in the case of mouse hepatoma.

SUMMARY AND CONCLUSIONS

The data presented show that the hydrogen-ion concentration in hepatic tumors is definitely higher than in normal liver. The pH in the hepatoma of the fasted rat is about 7.0, and in liver about 7.4. The pH of the hepatoma is lowered to 6.4 when the animal is supplied with excessive glucose, whereas the liver pH remains unchanged. However, the amount of carbohydrate ingested by a rat eating a balanced diet *ad libitum* is not enough to lower the tumor pH below that in the fasted rat. A tumor acidity corresponding to pH 6.4 appears to be the maximum attainable within functional limits. Hepatomas in mice present an analogous picture, except that the hydrogen-ion concentration is somewhat higher. The pH of hepatic tumors in fasted mice is about 6.7, and in mice injected with glucose 6.2.

The decrease in pH of the rat hepatoma upon glucose injection is caused, at least in part, by the formation of lactic acid during glycolysis, since the concentration of this metabolic product in the tumor is more than doubled by intraperitoneal injection of glucose. However, in the mouse hepatoma there is only a very small increase in lactic-acid concentration, and some other mechanism must be responsible for the increased acidity. Dubuisson (*16*) found that lactic acid alone would not account for the changes in pH of contracting isolated muscle but that phosphoric acid liberated from adenine triphosphate and creatine phosphate are partially responsible. Resynthesis of glycogen, a process involving organic phosphates, takes place in the mouse hepatoma as rapidly as in liver and may account for the inconsistency observed.

During the course of this investigation, it was found that rat hepatoma 31 could be transplanted into liver but that it grew very slowly.

[4] *Dr. Edwards examined these tissues for glycogen after they were fixed in alcohol and stained with Best's carmine.*

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Quantitative Analysis of Dose-Response Data Obtained With Three Carcinogenic Hydrocarbons in Strain C3H Male Mice

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In a previous communication (*1*) analyses were made of published data dealing with the responses of mice to measured quantities of several carcinogenic hydrocarbons. Certain conclusions were reached regarding the nature of the latent-period and percentage response curves obtained with such agents. This paper deals with investigations carried out to test further these conclusions and to compare the results obtained in simultaneous studies of 20-methylcholanthrene, 1, 2, 5, 6-dibenzanthracene, and 3, 4-benzpyrene.[1] The former conclusions are substantiated, and estimates are made of the relative carcinogenic potencies of the above-named materials.

EXPERIMENTAL PROCEDURE

The corrected melting points of the three hydrocarbons used were: methylcholanthrene, 179.7°–180.2° C.; dibenzanthracene, 267.3°–267.7°; benzpyrene, 178.2°–178.9°.[2] The same batches of the chemicals were used throughout the investigation.

The hydrocarbons were dissolved in tricaprylin with a melting point of 8.3°–9.0°. The various solutions used for injection were prepared by making appropriate dilutions of standard stock solutions of the hydrocarbons so that the unit volume of solvent containing the quantity of hydrocarbon injected was 0.25 cc., except in two dose groups, noted in the tables, where 0.5 cc. of solvent was used. The suspensions containing the higher concentrations were prepared by weighing separate batches of the hydrocarbons for each dose and adding the desired amount of solvent.

The doses were spaced logarithmically over a wide range (0.00024 to 8.0 mg.) in order to include extreme responses and thus obtain information regarding the range of doses within which both the incidence and the latent period of tumor response are correlated with dose.

The test animals were 1,004 male mice of strain C3H, which were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and which were received in varying lots during the first 3 months of 1941. Each lot was divided at random into 3 groups for injection with the respective hydrocarbons. Subdivision of the groups according to the various doses of the 3 chemicals was also carried out. The mice were 1.5 to 3 months old, and most of them weighed 20 to 24 gm. at the time of injection. They were maintained on unlimited supplies of water and Purina dog chow, and were individually identified.

All injections were made into the sub-

[1] *Hereinafter referred to as methylcholanthrene, dibenzanthracene, and benzpyrene.*

[2] *The hydrocarbons and the solvent were supplied by Dr. J. L. Hartwell, of the National Cancer Institute.*

cutaneous tissues of the right axilla. In order to prevent leakage, a long needle was inserted just above the tail in the dorsal region and passed diagonally through the subcutaneous tissues into the right axillary space. The volumes injected were measured with tuberculin syringes of 0.5-cc. capacity.

Examination, by palpation, of each individual mouse for subcutaneous tumor was carried out every fourth or fifth day beginning 4 weeks after injection. Mice with subcutaneous nodules thought to be early tumors were isolated at each examination, and the growth of the nodules was followed every second day by measurement until the growth had reached a mean diameter of about 20 mm. unless death intervened. Sections through several planes were taken of the tumor tissue at necropsy for microscopic examination.

A progressively growing tumor when proved by subsequent growth and histologic appearance to be a malignant neoplasm, was listed as having appeared on the date on which the nodule was first recorded. The interval between injection and appearance of the tumor was considered the latent period.

Two factors may complicate the first detection of a tumor nodule. The first is the formation of cysts containing the injected solvent. These cysts are ruptured by slight pressure and can be ruled out easily (*2*). The second factor is the thickening of the subcutaneous tissue which occurs with higher concentrations of the hydrocarbons (*3*). Occasionally the thickening may be nodular because of small encapsulated areas of necrosis or oil. This reaction appears fairly soon after injection and usually subsides before the appearance of a progressively growing malignant process.

Of the 1,004 strain C3H male mice originally employed, 957 contributed to the final analysis of results. Forty-seven animals were listed as missing or dead and autolyzed before small nodules were conclusively diagnosed as tumors and were discarded from the results.

A total of 433 tumors [3] at the site of injection was observed in the 957 mice. The morphology of 415 tumors that were examined histologically is described and analyzed in another publication (*4*). Of the 415 tumors, 411 (99 percent) were spindle-cell sarcomas, 2 were carcinomas, and 2 consisted of mixed sarcoma and carcinoma elements. The 18 tumors that have been included, in the absence of histologic examination, grew rapidly to a fairly large size (10 to 20 mm. in diameter) but were lost for microscopic studies because of death and autolysis or because they were devoured by other mice.

RESULTS

The results for the different hydrocarbons are summarized in tables 1, 4, and 7, respectively, which give as much information as is practicable concerning the actual data. Detailed information in regard to individual latent periods for the respective hydrocarbons is given in the scatter diagrams of figures 1, 9, and 18.

Analyses were made to determine the variability of the biologic responses and to determine the trends of the various group responses with dose of hydrocarbon. In addition, certain statistics were calculated to facilitate comparison between the results obtained with the various carcinogenic agents as well as with data from the literature. For simplicity, graphic presentation of the analyses is given where practicable. Most of the biomathematical procedures involved in these analyses are discussed in detail by Bliss (*5*, *6*, *7*) and

[3] *A few mice developed multiple tumors, but only the first tumor occurring in a given animal is considered in this report.*

Irwin (*8*). Where tests for statistical significance were carried out, the results are indicated in the text as follows: statistically significant, when P is less than 0.01; not statistically significant, when P is greater than 0.05; probably significant when P is between 0.01 and 0.05. Values preceded by a $\pm$ sign represent standard errors. Limits of error corresponding to a probability of 0.05 are designated where included.

Methylcholanthrene

The results with methylcholanthrene are summarized in table 1. The upper limit, 1.0 mg., of the dose range (0.00024 to 1.0 mg.) was selected because previous studies (*9*), in which methylcholanthrene had been administered to similar test animals under similar conditions, had showed that the incidence of tumors was 100 percent at all doses above 0.25 mg. Furthermore, above this dose level the latent period was constant and unrelated to dose.

TABLE 1.—*Summarized results on latent period of tumors and tumor incidence of mice injected with methylcholanthrene*

Dose [1]		Mice injected	Mice with tumors	Latent period			Tumor incidence			
				Mean latent period [2]	Standard deviation [2]	Weight of observation [3]	"Corrected total"	Mice with tumors	Probits [4]	Weight of observations
Mg.	*Log mg.*	*Number*	*Number*	*Months*	*Months*		*Number*	*Percent*		
1.0	0.000	20	20	2.40	0.40	20	20	100		
0.5	−.301	21	21	2.57	.40	21	21	100	[5] 8.484	0.063
0.25	−.602	21	21	2.77	.61	7.64	21	100	[5] 7.860	.441
0.125	−.903	21	21	3.32	.92	3.72	21	100	[5] 7.268	1.911
0.062	−1.204	21	17	3.92	1.01	1.78	19.7	86.3	6.094	7.98
0.031	−1.505	20	13	5.24	1.87	.90	19.9	65.3	5.393	11.96
0.0156	−1.806	18	6	4.59	.96	.29	16.5	36.4	4.652	9.97
0.0078	−2.107	17	3	6.97	4.64	.11	15.8	19.0	4.122	7.55
0.0039	−2.408	19	0	----	----	----	[6] 14.6	0	[5] 2.893	1.372
0.00195	−2.709	19	0	----	----	----	[6] 16.6	0	[5] 2.319	.578
0.00098	−3.010	41	0	----	----	----	[6] 29.2	0	[5] 1.695	.175
0.00024	−3.612	79	0	----	----	----	[6] 33.6	0	----	----

[1] The volume of solvent used for each injection was 0.5 cc. at dose 1.0 mg.; 0.25 cc. at all other doses.
[2] Months = Days/30.
[3] See text.
[4] See Bliss (*5, 7*).
[5] Estimated probits for 0- and 100-percent responses.
[6] Provisional values determined by extrapolation.

Latent Period

Standard deviation.—The latent periods are shown graphically in figure 1, in which they have been plotted against the logarithm of dose. The graph shows the variation of the latent periods at successive dose levels, i.e., the vertical spread of the plotted points. The horizontal and oblique lines represent calculated values and are for the purpose of showing the estimated vertical distribution at continuous dose levels. They are in terms of accumulative percentages of mice (within the tumor population only) which would be expected to develop tumors at or prior to the times indicated along the ordinate (fig. 1). For example, the top line of the figure shows for any given dose the time at which 99 percent of the mice that are going to develop tumors would be expected to have developed them. The derivation of the calculated lines is discussed later (p. 509).

In figure 1 it will be noted that the latent periods are most stable at the two highest doses (0.5 and 1.0 mg.), but that their variability increases as the dose becomes smaller.

The standard deviation of individual latent periods about their respective group

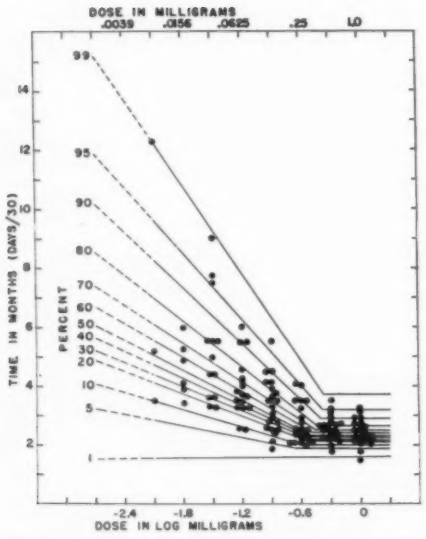

FIGURE 1.—Latent periods of tumors induced with methylcholanthrene. The solid circles represent individual observations plotted to avoid superposition of points at given doses. The oblique and horizontal lines show the calculated variation at continuous dose levels.

means are given in table 1 and shown graphically in figure 2. Their regression on log dose is statistically significant and

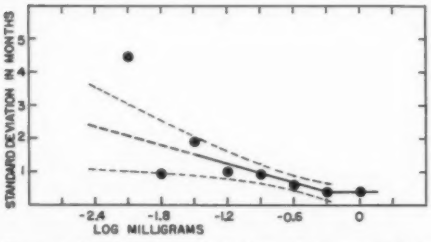

FIGURE 2.—Standard deviation of latent periods with successive doses of methylcholanthrene.
Circles: Observed standard deviations;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

may be adequately represented as linear within the limits indicated by the solid oblique line. The equation to the regression line is:

$$\sigma_c = 0.584 - 0.950\ (X + 0.519) \qquad (1)$$

where σ_c is the calculated standard deviation in months (days/30) and X the dosage in log milligrams. The curved lines[4] show the limits of error of the calculated regression line corresponding to $P=0.05$. The wide range of variation coupled with a low incidence of tumors at the low dose levels make estimates of the standard deviation in this region of little practical importance unless very large numbers of animals are used. The standard errors of the estimates for the two lowest doses (0.015 and 0.0078 mg.) are so large that the information which they contribute with respect to the true nature of the trend is of no consequence. The regression in this region is therefore indicated as a broken line which should be considered only as a provisional estimate and not as definite information regarding the exact nature of the trend.

On a basis of previous studies (*9*) in which the value of the minimal standard deviation was identical with that reported here, 0.4 months (days/30), the standard deviation is considered to have reached its minimal level at about dose 0.5 mg. (−0.301 log mg.). That the standard deviation of individual latent periods would not be decreased below this level by larger doses of methylcholanthrene is further indicated by the breaks in the trends of results shown in figures 1 and 2.

Relative weights of latent-period observations at different dose levels.—The reliability of a latent-period response, such as a group mean, is dependent upon the variability as well as upon the total number of individual observations employed in its derivation. In the following analyses, the

[4] *The curved lines of this and subsequent figures represent the limits within which the relation between dose and response has been established. They apply to the calculated regression line and not to the distribution of points about the calculated line.*

reciprocal of variance ($1/\sigma^2$) was used as a weighting factor for adjusting the weights of responses at different dose levels with respect to the variability factor.[5]

Table 2, column 5, shows the relative weights when the number of observations (mice with tumors) is constant and when the maximum weight for any one dose group is considered as unity. The maximum weight is reached with those doses that yield the minimal standard deviation. The relative weights may be employed as weighting coefficients, and they may also be used as a basis for estimating the numbers of unit observations required to give results possessing the same degree of reliability at different levels of dosage. Column 6 shows the relative numbers when 10 are considered at the dose level where the weight is at its maximum. It should be emphasized that the relative numbers in this column refer to individual latent-period determinations and that they represent, therefore, only mice that actually develop tumors. The incidence of tumors decreases with the dose of methylcholanthrene, and it is necessary to increase accordingly the total number of injected mice to produce the desired numbers with tumors indicated by column 6. (See section entitled "Joint Use of Latent Period and Tumor Incidence Data," also columns 7 and 8 of table 2.)

[5] *This procedure was suggested by Dr. Harold F. Dorn, Division of Public Health Methods, National Institute of Health, to compensate for the correlation between standard deviation and dose. In the present analyses, the calculated standard deviations, derived from equation (1), were used rather than the observed standard deviations. The maximum value of $1/\sigma_c^2$ was considered as unity for determining the relative weights, or weighting coefficients, used in the analyses.*

TABLE 2.—*Relative weights of latent-period observations and numbers of mice required for observations of equal weight at various doses of methylcholanthrene*

Dose (in milligrams)	Expected mean latent period [1]	Expected standard deviation [1] (σ_c)	Reciprocal of variance ($1/\sigma_c^2$)	Relative weight (weighting coefficient) [2]	Mice with tumors required for responses of equal weight	Expected incidence of tumors	Total injected mice required for latent period responses of equal weight
	Months	*Months*			*Number*	*Percent*	*Number*
1.0	2.48	0.40	6.25	1.0	10	100.0	10
0.5	2.48	.40	6.25	1.0	10	100.0	10
0.25	2.73	.66	2.27	.364	27	99.6	27
0.125	3.40	.95	1.11	.178	56	97.3	58
0.0625	4.07	1.23	.656	.105	95	88.1	108
0.0312	4.74	1.52	.432	.069	145	66.8	217
0.0156	5.42	[3] 1.81	[3] .306	[3] .049	[3] 204	37.9	[3] 538
0.0078	6.09	[3] 2.09	[3] .228	[3] .036	[3] 278	14.6	[3] 1,904
0.0039	6.76	[3] 2.38	[3] .177	[3] .028	[3] 357	3.6	.....

[1] Months = Days/30.
[2] See text.
[3] Provisional values determined by extrapolation.

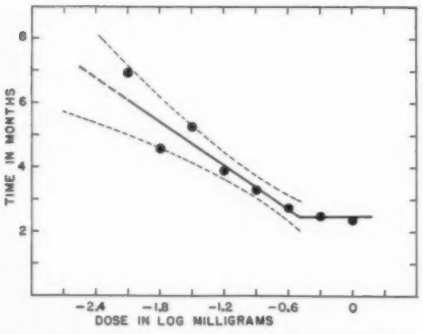

FIGURE 3.—Dose response curve for methylcholanthrene showing regression of mean latent period on log dose.
Circles: Observed mean latent periods;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

Mean latent period.—Statistical analyses [6] indicate that the regression of mean latent period on log dose is significant when dosages of methylcholanthrene smaller than 0.5 mg. (−0.301 log mg.) are used.

The regression (below dose −0.3 log mg.) is adequately represented by the oblique line of figure 3, the equation to which is:

$$Y=3.274-2.233\ (X+0.846) \qquad (2)$$

where Y is the estimated value of the mean latent period in days/30 and X the dosage in log milligrams.

The latent period is considered to have reached a minimal level with the two highest doses, 0.5 and 1.0 mg. (−0.3 and 0 log mg.). This is indicated by the apparent break in the trend of responses at this dose level (figs. 1 and 3) and by the results of a previous study (*9*) in which the established minimal latent period, 2.27 days/30, was very nearly the same as that found here, 2.48 days/30.

Frequency distribution of individual latent-period observations.—Exact information regarding the distribution of individual latent periods about the true response curve for methylcholanthrene cannot be derived from data available at the present time. However, an approximation made from the distribution of observed latent periods about their respective group means may be of value for certain practical purposes such as the weighting of animals that die without tumors, and estimation of the time-tumor frequency relationship at various dose levels.

In a previous communication (*1*) the frequency relationships of the normal curve were used for weighting animals that died without tumors.[7] It would be more desirable, however, to use the actual frequency distribution if this were known. Figure 4 shows the distribution of latent periods obtained by compiling the data of the present experiments and those of Shimkin (*11*) to give a total of 198 unit observations suitable for the analysis. Combination of the data for various doses and the different experiments was made possible by expressing the individual latent periods as deviations from their group means in standard deviation units.

It is apparent from figure 4 that the frequency distribution is fundamentally skewed, indicating an excess of values in

[6] *The finding in a previous section of a significant correlation between the standard deviation of individual latent periods and the logarithm of dose necessitates a modification of the statistical procedures described by Irwin* (8) *for analyses involving the continuous type of variate. Irwin treated only the condition in which the standard deviation is the same at all dose levels but pointed out that, if this were not the case, special procedures would have to be introduced. It is beyond the scope of the present investigation to deal with statistical techniques, and no attempt was made to work out special procedures for the latent-period data. An approximate method, which employed as the standard error of estimate the over-all average value for the standard deviation of group responses about the calculated regression line, was used in determining the limits of error and in making tests of significance.*

In computing the regression equation the variation in standard deviation with log dose was compensated for by weighting the latent-period observations with respect to the reciprocal of variance (see footnote 5) as well as in accordance with the number of observations.

[7] *The data treated were those of Lettinga* (10).

defect of the mean and a tendency to "tail out" toward the higher latent-period values. A better approximation to the observed frequencies would result, therefore, from use of the empirically established skew curve than from the relationships of a normal curve.

More suitable for present use is the accumulative form of the frequency curve shown in figure 5. The relationships between accumulative frequency and deviate represented by the curve of figure 5 are shown in actual time units (days/30) for the various dose levels by the solid (calculated) lines of figure 1. The transformation to actual time units was accomplished by adding (or subtracting) appropriate multiples of the expected standard deviation to (or from) the expected mean latent period for a given dose. The expected values were derived from the respective regression lines. (See figs. 2 and 3, or equations 1 and 2.) Accumulative percentages corresponding to various multiples of the standard deviation are shown along the ordinate (fig. 5).

The analysis of the frequency distribution was, of necessity, based on the combined data of all dose groups, and the possibility of a variation in degree of

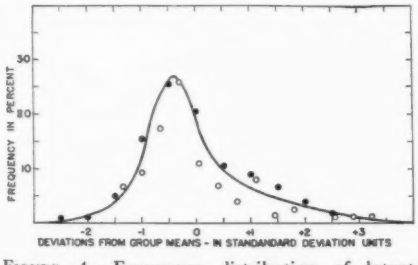

FIGURE 4.—Frequency distribution of latent-period responses obtained with methylcholanthrene, expressed as deviations from group means. The smoothed curve was drawn by sight.

Solid circles: Present data;
Open circles: Data of Shimkin (*11*).

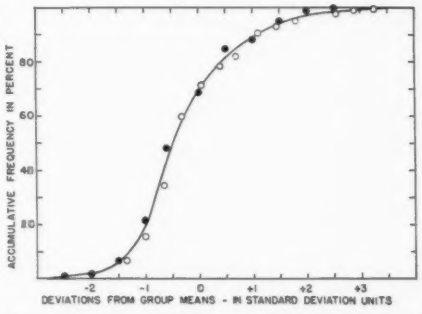

FIGURE 5.—Accumulative frequency curve of latent-period responses obtained with methylcholanthrene, expressed as deviations from group means. The smoothed curve was drawn by sight.

Solid circles: Present data;
Open circles: Data of Shimkin (*11*).

skewness with dose could not be investigated with the limited data available. The above frequency distribution, therefore, represents an over-all average. It has been used to express the results over the entire dose range pending the accumulation of sufficient data for a more detailed analysis. Another question of importance concerns the character of the frequency curve in the dose range where the latent-period response curve is flat, i. e., above 0.5 mg. (−0.301 log mg.). It is possible that the distribution at this dose level will be entirely different from that represented here (see analysis of dibenzanthracene data), but the available data are not sufficient for a separate analysis of this dose range.

Tumor Incidence

The incidence of tumors, in percent, was determined from the "corrected totals" (table 1). The procedures for estimating the corrected totals have been discussed in detail previously (*1*). Briefly, the method is that animals that die without tumors are weighted with respect to the proportion of animals in the tumor popu-

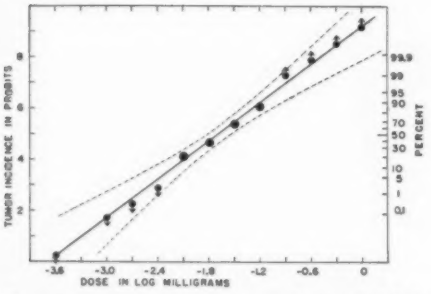

FIGURE 6.—Dose response curve for methylcholanthrene showing the regression of probits on log dose.

Circles: Empirical probits corresponding to observed percentages;
Circles with arrows: Estimated probit values for 0- and 100-percent responses;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

lation that would be expected to have developed tumors at or prior to the time of death of the animal under consideration.

Tumor incidence in relation to dose.—For a statistical evaluation of the data, the percentage values were converted into probits (table 1), and the doses to logarithms (table 1, log milligrams). The regression of probits on log dose is adequately described by a straight line (deviations of the observed values from a straight line are not statistically significant) thus indicating that the logarithms of individual effective doses of methylcholanthrene are normally distributed. The relations are shown in figure 6.

The regression equation is:

$$Y=5.098+2.474\ (X+1.641) \quad (3)$$

where Y is the estimated probit corresponding to dose X in log milligrams.

The percentage equivalents of the observed and calculated probits are given in figure 7. The S-shaped curve of this figure permits a better visualization of the practical dose limits within which the percentage response may be used for quantitative studies. Without taking into consideration the limits of error of the estimated curve, the useful dose range is seen to be from about 0.0045 mg., the dose calculated to give tumors in 5 percent of the mice (TD_5), to about 0.096 mg., the dose which would be expected to yield a 95-percent incidence of tumors (TD_{95}). Beyond these limits the responses rapidly approach 0 and 100 percent, respectively, and there is no further correlation between dose and tumor incidence.

Median tumor dose (TD_{50}).—The dose of methylcholanthrene which would be expected on a basis of the present data to produce tumors in 50 percent of the test animals is -1.681 ± 0.063 log mg., or $0.021^{+0.0032}_{-0.0028}$ mg. The limits of error corresponding to $P=0.05$ are ±0.123 log mg., or $^{+0.0068}_{-0.0051}$ mg.

Standard deviation of the logarithms of individual effective doses about log TD_{50}.—Gaddum (*12*) has shown that the S-shaped dose-response curve may be considered as an integrated frequency curve of individual effective doses and that the standard de-

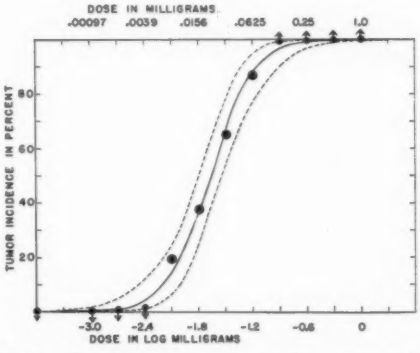

FIGURE 7.—Dose response curve for methylcholanthrene showing relation of tumor incidence in percent to log dose. Transformed from data of figure 6.

viation, λ, of the logarithms of individual effective doses about the median effective dose may be estimated from this curve or from its counterpart in probability units, e. g., probits (*5*), or normal equivalent deviations (*12*). In the instance of methylcholanthrene, the value of λ estimated from probit data is 0.40 ± 0.06. The limits of error corresponding to $P=0.05$ are ± 0.13.

The statistic λ has been widely used in recent years for comparing the biologic actions of various drugs. It has also been used for comparing the responses of different types of test animals to a common material. Thus, Boyland and Warren (*13*) reported λ values of 0.58 and 0.73, respectively, for mice of strains CBA and Simpson which were injected subcutaneously with a lard solution of methylcholanthrene. The present estimate of the standard deviation, 0.40, is less than the values found by Boyland and Warren, but it is still within the limits for "normal drug action" as quoted by these authors, i. e., 0.37 to 0.91. It would appear, then, that the action of tricaprylin solutions of methycholanthrene in C3H male mice is less variable than that of lard solutions in CBA and Simpson strain mice.

Relative weights of tumor-incidence responses at different dose levels.—The reliability of a dosage, or potency, estimate inferred from a percentage (or probit) response varies according to the magnitude of the response itself, as well as with the number of unit observations on which it is based. Weighting coefficients for use with percentage (or probit) responses of various magnitudes have been published by Gaddum (*12, fig. 9*) and by Bliss (*5, table III*). The appropriate values corresponding to the expected percentage responses for methylcholanthrene are given in table 3, column 4. The relative numbers of mice that would be required to give percentage responses of equal weight, with respect to estimates of potency (table 3), are those which would be required if 10 were used at the 50-percent level.

TABLE 3.—*Relative weights of tumor-incidence observations and numbers of mice required for observations of equal weight at various doses of methylcholanthrene*

Dose (in milligrams)	Expected probit	Expected percent	Weighting coefficient [1]	Mice required for equal weight responses
				Number
1.0	9.158	100.0	0.000	.....
0.5	8.413	99.97	.004	1,667
0.25	7.669	99.62	.032	200
0.125	6.924	97.3	.148	43
0.062	6.179	88.1	.378	17
0.031	5.435	66.8	.594	11
0.0156	4.690	37.9	.614	10
0.0078	3.945	14.6	.420	15
0.0039	3.201	3.6	.180	35
0.00195	2.456	.55	.045	141
0.00098	1.711	.05	.006	1,111
0.000244	.222	0	.000	.....

[1] From Bliss (*5, table III*).

Joint Use of Latent-Period and Tumor-Incidence Data

Total numbers of injected mice required to give latent-period responses of equal weight at various dose levels.—The numbers of mice with tumors that would be required to produce latent-period responses of equal weight are given in table 2. It has been shown that the frequency of tumors among injected animals decreases progressively with the dose of methylcholanthrene (figs. 6 and 7). In order, therefore, to obtain a desired number of mice with tumors at a given dose level, it would be necessary to increase the total number injected to allow for those that fail to develop tumors. The expected tumor frequencies and the total numbers of injected mice based thereon are also given in table 2. The total numbers (column 8) represent approximations only, since the relations on which they are based are subject to variation. Account has not been taken of mice that die during the course of the experiment.

Specific induction time.—The specific induc-

tion time was previously defined (*1*) as the time required, on an average, for the production of tumors with dose TD_{50}. The value of this measure for comparing the relative rates of action of different carcinogenic hydrocarbons has already been emphasized (*1*). The specific induction time for methylcholanthrene is 5.14 ± 0.55 months (days/30).[8] The limits of error corresponding to $P=0.05$ are ± 1.05 months (days/30).

Time-tumor freauency relationships at successive dose levels.—Time-frequency curves have been widely used in presenting the results obtained with single doses of carcinogenic hydrocarbons or in comparing the results obtained with two or more preparations of such materials. This procedure gives detailed information regarding the latent periods of individual animals of a group and offers the advantage that the responses to various preparations may be compared on a common basis at any particular time during the course of an experiment. Thus, the essential information of an experiment may be obtained at a time when most of the tumors have appeared, e. g., 9 months, but several months before full opportunity has been allowed for the extreme cases to develop, e. g., 12 to 18 months. On the other hand, quantitative determinations of the potency relationships between various preparations of a carcinogenic agent are simplest when a maximum time has been allowed for tumors to appear and when the estimates are based on final percentage observations, i. e., on observations which are no longer changing with time. Potency relationships may, nevertheless, be established from observations made during the period when tumors are still appearing provided the time-frequency relationship is known for successive levels of potency (or dosage) and a series of standard curves is available for comparison. Such a series of standard curves may be constructed from the latent-period and tumor-incidence relationships presented previously. For example, the percentage of mice in the tumor population which would be expected to develop tumors at, or prior to, a given time is shown for various levels of dosage by the calculated lines of figure 1. These percentages may be converted to percentages of the total injected population, and the time frequency relationship expressed in terms of the total numbers of injected mice in each dose group.

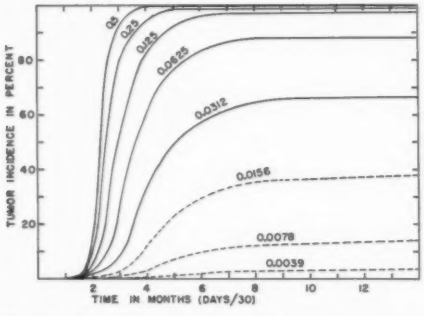

FIGURE 8.—Time-frequency curves for successive doses of methylcholanthrene showing the calculated incidence of tumors at successive periods.

The time-frequency curves for the various doses of methylcholanthrene are shown in figure 8. Although this series of curves illustrates the nature of the time-frequency results to be expected at different levels of dosage and while it may be profitably used in the planning of future experiments under the present conditions, it cannot at present be considered as a standard for quantitative comparison. For the latter usage it would be necessary to prove (1) that this estimate is fairly close to the true results that would be obtained with very

[8] *The value 5.14 was obtained by substituting -1.681, log TD_{50}, for X in equation 2 and solving for Y, the expected latent period at this dose level. The limits of error were estimated by taking into consideration the error of estimate of log TD_{50} as well as the error of the latent period regression equation.*

large numbers of observations, and (2) that the animal population remains relatively stable over long periods of time. No attempt has been made to set the limits of error of the curves of figure 8, since involved in their estimation are errors of the latent-period response curve, errors of the probit response curve, errors of the standard-deviation regression line, and finally errors of estimate of the latent-period frequency distribution.

In spite of wide possible limits of error of the curves of figure 8, some information concerning their nature has been gained. For example, it is apparent that tumors may occur practically as early with the lower doses as with the higher doses of methylcholanthrene although the probability of early tumors becomes less as the dose is decreased. Furthermore, the curves asymptote at various levels below 100 percent as the dose is decreased below about 0.5 mg., and an incidence of 100 percent would never be reached at these low doses regardless of the length of time the observations are continued.

Dibenzanthracene

The summarized results obtained with dibenzanthracene in doses of 0.00195 to 8.0 mg. are presented in table 4. The discussion concerning analytical procedures in the section on methylcholanthrene is applicable also to dibenzanthracene, and the reader is referred to that section for explanation of the methods of presentation and the purposes of the various analyses.

TABLE 4.—*Summarized results obtained on latent period of tumors and tumor incidence of mice injected with dibenzanthracene*

Dose [1]		Mice injected	Mice with tumors	Latent period			Tumor incidence			
				Mean latent period [2]	Standard deviation [2]	Weight of observation [3]	"Corrected total" [3]	Mice with tumors	Probits [4]	Weight of observation
Mg.	*Log mg.*	*Number*	*Number*	*Months*	*Months*		*Number*	*Percent*		
8.0	+0.903	21	16	3.75	0.32	16.0	16.0	100.0		
4.0	+.602	20	17	3.83	.62	17.0	18.6	91.5	6.372	
2.0	+.301	19	19	3.69	.64	19.0	19.0	100.0		
1.0	0	22	22	3.60	.54	22.0	22.0	100.0		
0.5	−.301	21	20	3.76	.56	15.4	20.1	99.5		
0.25	−.602	21	19	4.01	1.16	7.11	20.0	95.0	6.645	
0.125	−.903	23	21	4.47	1.48	4.62	21.9	95.9	6.739	4.58
0.062	−1.204	20	20	5.10	1.91	2.90	20.0	100.0	[5] 6.726	8.46
0.031	−1.505	21	16	6.31	1.67	1.63	19.8	80.8	5.870	11.44
0.0156	−1.806	19	6	5.94	1.41	.46	15.8	38.0	4.694	10.05
0.0078	−2.107	40	6	8.78	2.61	.35	32.1	18.7	4.111	17.30
0.00195	−2.709	79	2	9.48	2.40	.08	61.9	3.2	3.148	10.27

[1] The volume of solvent used for each injection was 0.5 cc. at dose 8.0 mg.; 0.25 cc. at all other doses.
[2] Months = Days/30.
[3] See text.
[4] See Bliss (*5, 7*).
[5] Estimated probity for the 100-percent response.

Latent Period

Standard deviation.—Inspection of figure 9 reveals that above dose 0.5 mg. the variability of individual latent periods is relatively stable whereas below this dose variability increases progressively as the dose is decreased. The standard deviations of individual latent periods obtained with different doses are listed in table 4 and are shown graphically in figure 10. The regression of standard deviation on log dose is probably significant in the range 0.00195 to 0.5 mg., but there is no correlation between standard deviation and

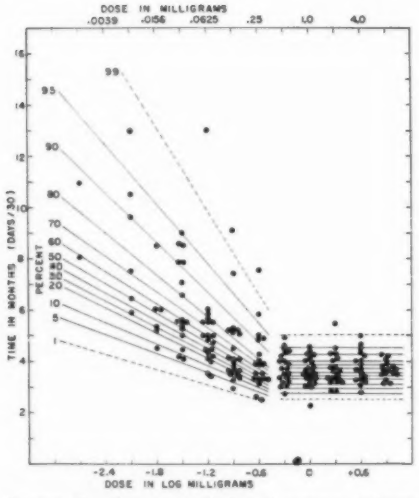

FIGURE 9.—Latent periods of tumors induced with dibenzanthracene. Solid circles represent individual observations plotted to avoid superposition of points at given doses. The oblique and horizontal lines show the calculated variation at continuous dose levels.

dose in the range 1.0 to 8.0 mg. The regression in the lower dose region may be represented as linear as shown by the oblique line (fig. 10). The equation to this line is:

$$\sigma_c = 0.935 - 0.894\ (X + 0.649) \qquad (4)$$

where σ_c represents the calculated value of the standard deviation in months (days/30) and X the dose in log milligrams. It is apparent that at about dose 0.5 mg. (−0.301 log mg.) the standard deviation reaches a minimal level below which it is not further reduced in spite of progressive increases in dosage. On the other hand, with doses of dibenzanthracene smaller than 0.5 mg. the standard deviation becomes progressively greater as the dosage is decreased.

Relative weights of latent-period responses at different dose levels.—The relative weights of latent-period observations when equal numbers of tumorous mice are available for the successive doses of dibenzanthracene are shown in table 5. They represent the relative weights when the maximum weight is considered as unity.

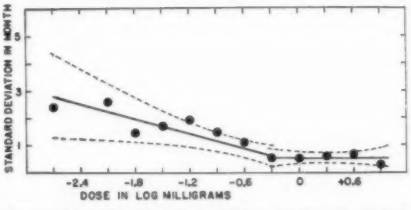

FIGURE 10.—Standard deviation of latent periods with successive doses of dibenzanthracene.
Circles: Observed standard deviations;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$

TABLE 5.—*Relative weights of latent-period observations and numbers of mice required for observations of equal weight at various doses of dibenzanthracene*

Dose (in milligrams)	Expected mean latent period [1]	Expected standard deviation [1] (σ_c)	Reciprocal of variance ($1/\sigma_c^2$)	Relative weight (weighting coefficient) [2]	Mice with tumors required for responses of equal weight	Expected incidence of tumors	Total injected mice required for latent period responses of equal weight
	Months	*Months*			*Number*	*Percent*	*Number*
8.0	3.72	0.544	3.38	1.0	10	90 to 100	10 or 11
4.0	3.72	.544	3.38	1.0	10	90 to 100	10 or 11
2.0	3.72	.544	3.38	1.0	10	90 to 100	10 or 11
1.0	3.72	.544	3.38	1.0	10	90 to 100	10 or 11
0.5	3.72	.624	2.57	.760	13	90 to 100	13 or 14
0.25	3.88	.893	1.25	.370	27	90 to 100	27 to 29
0.125	4.61	1.16	.743	.220	45	90 to 100	45 to 48
0.062	5.34	1.43	.489	.145	69	91.2	76
0.031	6.08	1.70	.346	.102	98	74.8	131
0.0156	6.81	1.97	.258	.076	132	49.4	267
0.0078	7.54	2.24	.199	.059	169	24.2	698
0.0039	8.27	2.51	.159	.047	213	8.3	2,566
0.00195	9.00	2.78	.129	.038	263	1.9	.....

[1] Months = Days/30.

[2] See text.

The relative numbers of mice with tumors required to give latent-period responses of equal weight when 10 are used at the level of maximum weight are shown in table 5, also the total numbers required to give the desired numbers with tumors. (See section entitled "Joint Use of Latent Period and Tumor-Incidence Data.")

Mean latent period.—The mean latent periods for the various doses of dibenzanthracene are listed in table 4, colum 5; their trend with log dose is shown graphically in figure 11. There is a statistically significant regression of mean latent periods on log dose within the dose range 0.00195 to 0.25 but not within the range 0.5 to 8.0 mg. The responses within the latter dose limits undoubtedly represent the minimal limiting level of the latent period for dibenzanthracene under the present condition. The average value for the minimal latent period, represented by the horizontal line (fig. 11) is 3.72 months (days/30) which is identical with that found for dibenzanthracene in previous analyses (*1*) of the data of Lettinga (*10*).

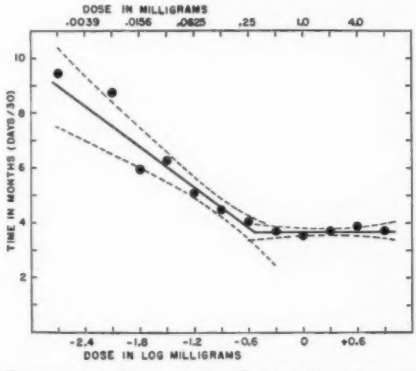

FIGURE 11.—Dose response curve for dibenzanthracene, showing regression of mean latent period on log dose.

Circles: Observed mean latent periods;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

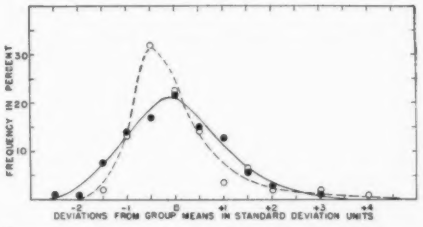

FIGURE 12.—Frequency distributions of latent-period responses obtained with dibenzanthracene, expressed as deviations from group means. The smoothed curves were drawn by sight.

Solid circles: Data of dose groups 0.5 to 8.0 mg.;
Open circles: Data of dose groups 0.00195 to 0.25 mg.

The regression of mean latent periods in the dose range 0.00195 to 0.25 mg. is represented by the oblique line (fig. 11), the equation to which is

$$Y=4.709-2.427\ (X+0.942) \qquad (5)$$

where Y represents the estimated value of the mean latent period in months (days/30) and X the dosage in log milligrams.

Frequency distribution of individual latent-period observations.—In order to obtain sufficient data for an analysis of the frequency distribution, it was necessary to combine the data of the various dose groups. For this purpose the variates were expressed as deviations from group means in standard deviation units. Since it was found that the trend of the mean responses could be divided into two components, the data corresponding to the oblique and horizontal components of the response curve of figure 11 were compiled in separate lots. The frequency distribution curve obtained with the combined data of dose groups 0.00195 to 0.25 mg. is shown by the broken curve of figure 12. The solid curve shows the distribution for dose groups 0.5 to 8.0 mg. The accumulative forms of the frequency curves are shown in like manner in figure 13.

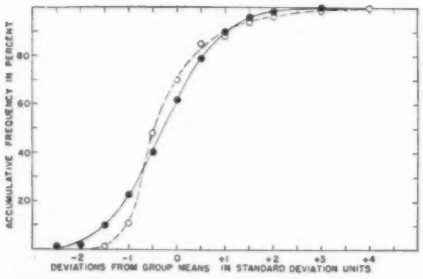

FIGURE 13.—Accumulative frequency curves of latent-period responses obtained with dibenzanthracene, expressed as deviations from group means. The smoothed curves were drawn by sight.

Solid circles: Data of dose groups 0.5 to 8.0 mg.;

Open circles: Data of dose groups 0.00195 to 0.25 mg.

The relations between accumulative frequency in percent, and deviate in standard deviation units, obtained by reference to the smoothed curves of figure 13, were employed in determining the expected accumulative frequency levels indicated by the calculated lines of figure 9. (See discussion of similar determinations with methylcholanthrene.)

Tumor Incidence

The corrected totals and the tumor-incidence responses obtained with dibenzanthracene are given in table 4. The empirical probits corresponding to the percentage estimates are also listed. The response to dose 4.0 mg. (0.6 log mg.) is considerably out of line with the trend of the remainder of the data when plotted in probability units (probits) (fig. 14). A similar phenomenon was noted in an earlier analysis (*1*) of the data of Lettinga (*10*), and, following the same procedure of analysis, this apparent discordant result was omitted in making the first approximation to the trend of the results. The computed regression line (solid line) is steeper than the apparent trend (dash line). The procedure of curve fitting developed by Bliss (7) for application to data on small numbers of animals, or data that are relatively variable about the smoothed trend, involves a series of approximations, using for each new approximation the preceding computed curve for correcting the probit values and their relative weights. In the present analysis a series of such approximations was carried out, and with each new approximation the corrected probit for dose 0.25 mg. (−0.602 log mg.) decreased progressively whereas the corrected values for the other empirical probits became practically identical with the empirical values themselves. Also with each new approximation, the estimated weight of the response to dose 0.25 mg., as estimated from the computed regression line, grew smaller, and the slope of the regression line became steeper, approaching the oblique line shown in figure 15. The low corrected probit for dose 0.25 mg. when considered together with the discordant result previously mentioned for dose 4.0 mg. indicated that the

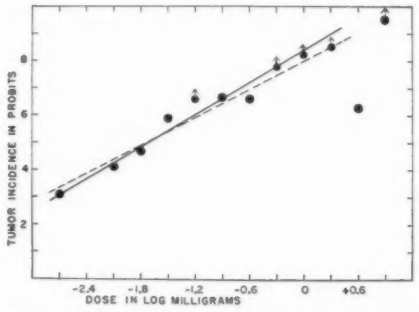

FIGURE 14.—Probit-dose relations obtained with dibenzanthracene.

Circles: Empirical probits corresponding to observed percentages;

Circles with arrows: Estimated probit values corresponding to 100-percent responses;

Dash line: Provisional regression line, drawn by sight;

Solid line: First calculated approximation to regression line.

probit-dose relationship for the higher doses of dibenzanthracene might actually be different from that obtained with lower doses. This was further indicated by the findings of Shimkin and Andervont (*9*) who studied the dose range 0.25 to 3.0 mg. under conditions similar to those of the present experiments. Their results all fell between 90 and 95 percent and were not correlated with dose. Additional evidence of erratic results with higher doses of dibenzanthracene is contained in the results of Lettinga (*10*), previously discussed in this connection (*1*), as well as in some unpublished data of Shear.[9] In view of these findings it seemed advisable to consider 0.125 mg. as the upper limit of the significant dose range and to determine the probit-dose relationship only in the dose region below this level. Accordingly, the response curve for dibenzanthracene was recalculated by using only the data of dose groups 0.00195 to 0.125 mg. The curve is shown in figure 15. The regression equation is:

$$Y=4.919+2.272\ (X+1.835) \qquad (6)$$

where Y is the expected incidence of tumors in probits and X is the dosage in log milligrams. The relations of figure 15 are shown in terms of percent in figure 16. The horizontal line, indicating a break at about dose 0.25 mg., shows the average level of the erratic responses.

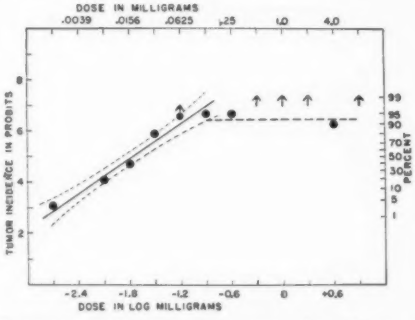

FIGURE 15.—Dose response curve for dibenzanthracene showing the regression of probits on log dose.
Circles: Empirical probits corresponding to observed percentages;
Circle with arrow: Estimated probit value for 100-percent response;
Arrows: Arrows designate doses that gave 100-percent responses in the region where the results were erratic;
Solid line: Calculated regression line for dose range 0.00195 to 0.125 mg.;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$;
Horizontal dash line: Average level of erratic responses in dose region 0.25 to 8.0 mg.

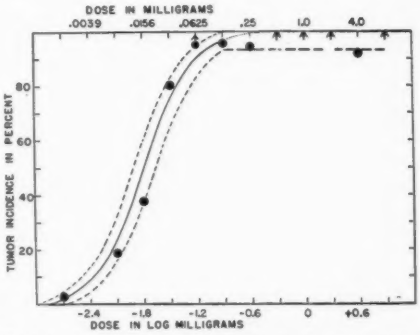

FIGURE 16.—Dose response curve for dibenzanthracene, showing relation of tumor incidence in percent to log dose. Transformed from data of figure 15. The dotted extension of the S-shaped curve shows the relations that would be expected to hold if the responses to the higher doses had followed the same trend as those of the lower doses.

The significant dose range for dibenzanthracene is from about 0.003 mg. (dose TD_5) to about 0.084 mg. (dose TD_{95}). The break in the probit response curve does not alter appreciably the useful dose range.

Median tumor dose (TD_{50}).—The dose of dibenzanthracene that would be expected to produce tumors in 50 percent of the injected animals was estimated from the present data to be -1.799 ± 0.056 log mg. The limits of error of the estimate corresponding to $P=0.05$ are ±0.11. In terms of milligrams, TD_{50} is, therefore,

[9] *Personal communication.*

$0.016^{+0.0022}_{-0.0019}$, and the limits corresponding to $P=0.05$ are $^{+0.0046}_{-0.0035}$.

Standard deviation of logarithms of individual effective doses about log TD_{50}.—The standard deviation, λ, of the logarithms of individual effective doses found from the present studies on dibenzanthracene, is 0.44 ± 0.05. The limits of error for $P=0.05$ are ± 0.094. This value does not differ significantly from tnat for methylcholanthrene in the preceding section, 0.40.

Relative weights of tumor-incidence responses at different dose levels.—Table 6 gives the expected probits and their equivalents in percent, for the various doses of dibenzanthracene. The values were computed from equation 6. The weighting coefficients were determined by reference to the tabled values published by Bliss. They represent the relative weights of the various percentage responses when the number of animals is the same in each dose group. The relative numbers of mice required to give responses of equal weight when 10 are used at the level of the 50-percent response (at dose TD_{50}) are presented in the last column of table 6.

Joint Use of Latent-Period and Tumor-Incidence Data

Total numbers of injected mice required to give latent-period responses of equal weight at various dose levels.—The relative numbers of mice with tumors required for observations of equal weight have been discussed in a preceding section. They are listed in table 5. The decreasing incidence of tumor-bearing mice with successively smaller doses of dibenzanthracene is shown in column 7 of table 5. The values represented are the expected percentages derived from the dose-response relationships developed (figs. 15 and 16). The last column shows the total numbers of mice that would have to be injected in order to obtain the desired numbers with tumors indicated by column 6.

TABLE 6.—*Relative weights of tumor-incidence observations and numbers of mice required for observations of equal weight at various doses of dibenzanthracene*

Dose (in milligrams)	Expected probit	Expected percent	Weighting coefficient	Mice required for equal weight responses
				Number
8.0 to 0.125	6.282 or greater.	90 to 100		
0.0625	6.353	91.2	0.318	20
0.0312	5.669	74.8	.540	12
0.0156	4.985	49.4	.636	10
0.00781	4.301	24.2	.532	12
0.00390	3.617	8.3	.302	21
0.00195	2.933	1.9	.117	54

Specific induction time.—The specific induction time is 6.79 ± 0.45 months (days/30). The limits of error corresponding to $P=0.05$ are ± 1.06.

The value 6.79 is identical with a previous estimate (*1*) of the specific induction time made from Lettinga's data on dibenzanthracene (*10*).

Time-tumor frequency relationships at successive dose levels.—The time-frequency curves for various doses of dibenzanthracene are shown in figure 17. They were derived, as described in the section on methylcholanthrene, by joint use of the calculated regression lines for both the latent-period and tumor-incidence types

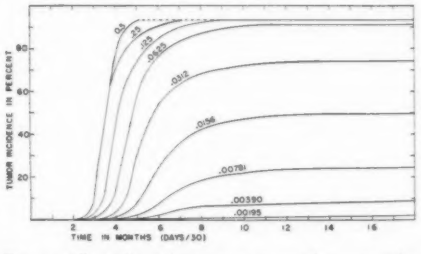

FIGURE 17.—Time frequency curves for successive doses of dibenzanthracene showing the calculated incidence of tumors of successive periods.

of responses as well as the estimated accumulative frequency curves of figure 13. As pointed out in the previous section, the curves are presented as rough approximations only, but they may serve as a guide in the planning of future experiments under these same conditions.

BENZPYRENE

The methods of presentation and the purposes of the analyses of benzpyrene data are similar to those in the preceding sections on methylcholanthrene and dibenzanthracene. Table 7 shows the summarized results obtained with doses ranging from 0.00195 to 8.0 mg. of the hydrocarbon.

Latent Period

Standard deviation.—As in the case of the other hydrocarbons, the results with benzpyrene show a statistically significant correlation between dose and the standard deviation of individual latent periods at all doses below a certain level, but a lack of correlation in the higher dose range. The spread of the individual latent-period observations is illustrated in figure 18. The standard deviations are shown in table 7 and their regression on log dose in figure 19. The relatively low value observed for dose 0.00195 mg. (−2.709 log mg.), was based on only two tumors and hence does not represent a satisfactory estimate. The extension

TABLE 7.—*Summarized results obtained on latent period of tumors and tumor incidence of mice injected with benzpyrene*

Dose [1]		Mice injected	Mice with tumors	Latent period			Tumor incidence			
				Mean latent period [2]	Standard deviation [2]	Weight of observation [3]	"Corrected total" [3]	Incidence of tumors	Probits [4]	Weight of observation
Mg.	*Log mg.*	*Number*	*Number*	*Months*	*Months*		*Number*	*Percent*		
8.0	+0.903	21	20	2.96	0.34	20	20.1	99.5	[5] 8.558	0.141
4.0	+ .602	19	16	3.02	.28	16	16.0	100.0	[5] 8.084	.432
2.0	+ .301	19	19	3.09	.39	14.33	19.0	100.0	[5] 7.626	1.615
1.0	0	20	18	3.32	.62	5.38	19.0	94.7	6.616	3.838
0.5	− .301	19	19	3.86	.83	3.02	19.0	100.0	[5] 6.796	7.011
0.25	− .602	21	14	4.41	1.15	1.39	21.0	66.7	5.432	11.235
0.125	− .903	19	15	5.11	1.49	1.00	18.0	83.3	5.966	11.322
0.062	−1.204	20	4	5.79	1.80	.20	19.8	20.2	4.165	12.038
0.031	−1.505	16	0				15.2	0	[5] 3.428	7.326
0.0156	−1.806	19	0				17.1	0	[5] 3.073	5.250
0.0078	−2.107	40	0				34.4	0	[5] 2.660	5.298
0.00195	−2.709	81	2	8.37	1.32	[6] .03	69.6	2.87	3.102	1.183

[1] Volume of solvent used for each injection was 0.25 cc. at all doses.
[2] Months = Days/30.
[3] See text.
[4] See Bliss (*5, 7*).
[5] Estimated probits for 0- and 100-percent responses.
[6] Provisional value.

of the regression line to this low dose level must therefore be considered as provisional. The extension is shown in figure 19 as a broken line. The regression equation is:

$$\sigma_c = 0.584 - 0.771\ (X - 0.056) \qquad (7)$$

where σ_c is the estimated standard deviation in days/30 and X the dose in log milligrams.

The minimal standard deviation was reached at about 2.0 mg. (0.3 log mg.). The average minimal standard deviation was 0.343 months (days/30).

Relative weights of latent-period observations at different dose levels.—The relative weights of latent-period observations with benzpyrene represent the relative weights when the maximum weight is considered as unity and when the number of mice with tumors is the same at all dose levels. The data are given in table 8.

TABLE 8.—*Relative weights of latent-period observations and numbers of mice required for observations of equal weight at various doses of benzpyrene*

Dose (in milligrams)	Expected mean latent period [1]	Expected standard deviation [1] (σ_e)	Reciprocal of variance ($1/\sigma_e^2$)	Relative weight (weighting coefficient) [2]	Mice with tumors required for responses of equal weight	Expected incidence of tumors	Total injected mice required for latent period responses of equal weight
	Months	*Months*			*Number*	*Percent*	*Number*
8.0	3.02	0.343	8.50	1.0	10	100.0	10
4.0	3.02	.343	8.50	1.0	10	99.7	10
2.0	3.02	.395	6.41	.754	13	98.8	13
1.0	3.30	.627	2.54	.299	33	96.0	34
0.5	3.89	.859	1.35	.159	63	88.8	71
0.25	4.47	1.09	.840	.099	101	75.6	134
0.125	5.05	1.32	.571	.067	149	56.5	264
0.062	5.63	1.56	.413	.049	204	35.8	570
0.031	[2] 6.21	[2] 1.79	[2] .313	[2] .037	[2] 270	18.6	[2] 1,452
0.0156	[2] 6.79	[2] 2.02	[2] .245	[2] .029	[2] 345	7.8	.....
0.0078	[2] 7.37	[2] 2.25	[2] .197	[2] .023	[2] 435	.7	.....
0.0039	[2] 7.95	[2] 2.48	[2] .162	[2] .019	[2] 526	.1	.....
0.00195	[2] 8.54	[2] 2.72	[2] .135	[2] .016	[2] 625	0	.....

[1] Months = Days/30.

[2] Provisional values.

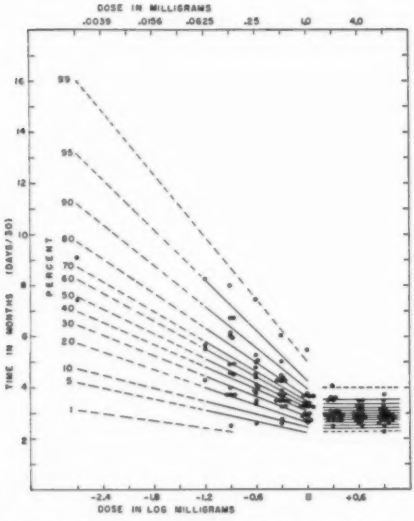

FIGURE 18.—Latent periods of tumors induced with benzpyrene. The solid circles represent individual observations plotted to avoid superposition of points at given doses. The oblique and horizontal lines show the calculated variation at continuous dose levels.

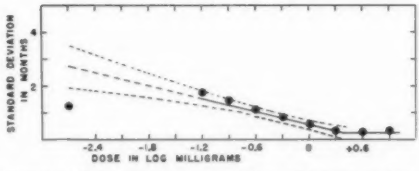

FIGURE 19.—Standard deviation of latent periods with successive doses of benzpy
Circles: Observed standard deviations;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

The relative numbers of mice with tumors required to give latent-period responses of equal weight are those which pertain when 10 are employed at the level of maximum weight. The total numbers of injected mice required to give the desired numbers with tumors (table 8) are discussed later in the section, Joint Use of Latent-Period and Tumor-Incidence Data.

Mean latent period.—In the dose region below 2.0 mg. the mean latent period increased significantly as the dose was decreased. The observed results gave an excellent fit to a straight line when plotted against the logarithm of dose (fig. 20). The mean latent period for dose 0.00195 mg. was based on only two unit observations and therefore does not represent a reliable estimate. It is fortuitous that it falls so close to the computed regression line which has been extended as a broken

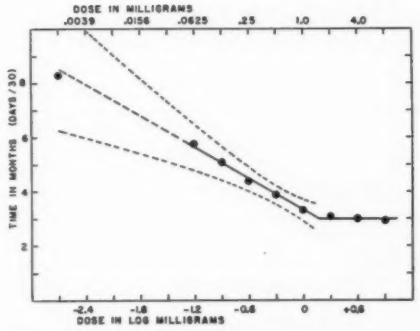

FIGURE 20.—Dose response curve for benzpyrene, showing regression of mean latent period on log dose.

Circles: Observed mean latent periods;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

line to the low dose region.[10] The regression equation is:

$$Y=3.826-1.931\ (X+0.270) \quad (8)$$

where Y represents the calculated value of the mean latent period in months (days/30) and X the dose in log milligrams.

As already noted, the mean latent period was not correlated with dose in the region 2.0 to 8.0 mg. This is shown by the break in the plotted results in figures 18 and 20. The average value of the minimal latent period was 3.02 months (days/30). It is shown graphically by the horizontal component of the response curve of figure 20.

Frequency distribution of individual latent-period observations.—As in the case of methylcholanthrene, the frequency distribution of individual latent periods was

[10] *The standard deviation of the mean latent periods about the calculated regression line (the error of estimate used for purposes of statistical analysis (see footnote 6)) was much less for benzpyrene than for the other hydrocarbons. That this result is fortuitous is indicated by the fact that figures 1, 9, and 18 show a similar degree of scatter of the individual latent periods. It was decided, therefore, to use as the error of estimate for the benzpyrene data the average of the values found for all three materials rather than the lower value calculated from the benzpyrene results alone.*

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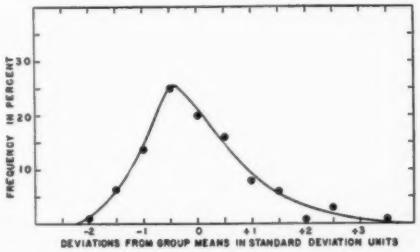

FIGURE 21.—Frequency distribution of latent-period responses obtained with benzpyrene, expressed as deviations from group means. The circles represent observed frequencies. The smoothed curve was drawn by sight.

determined from the combined data of all dose groups. Sufficient data were not available for a separate analysis of the dose groups corresponding to the horizontal component of the response curve. As in the previous analyses, combination of the data was made possible by conversion of the individual variates to deviations from group means in standard deviation units.

The frequency distribution based on the combined data is shown in figure 21, and the accumulative frequency curve in figure 22. The curve of figure 22 was used (as described in section on methylcholanthrene) for deriving the calculated lines of figure 18.

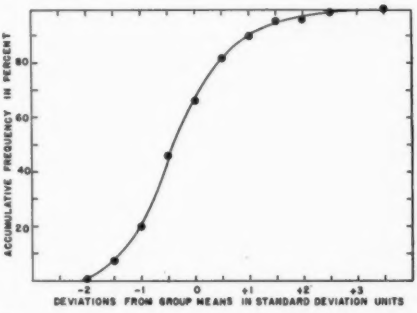

FIGURE 22.—Accumulative frequency curve of latent-period responses obtained with benzpyrene, expressed as deviations from group means. The circles represent observed frequencies. The smoothed curve was drawn by sight.

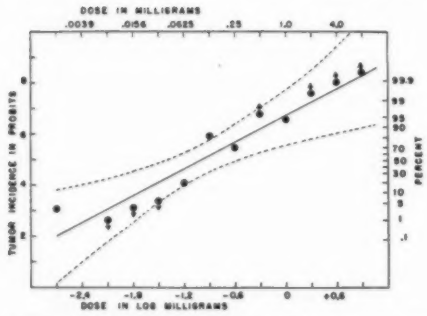

FIGURE 23.—Dose response curve for benzpyrene showing the regression of probits on log dose.

Circles: Empirical probits corresponding to observed percentages;
Circles with arrows: Estimated probit values for 0– and 100– percent responses;
Solid line: Calculated regression line;
Curved dash lines: Limits of error of calculated regression line for $P=0.05$.

Tumor Incidence

The summarized tumor-incidence results are presented in table 7. The observed percentages were converted to probits for the purpose of statistical analysis. The probit values given in table 7 are graphically represented by the solid circles of figure 23. The solid line of figure 23 represents the third computed approximation derived by the method of Bliss (7). It will be noted that two of the plotted points deviate considerably from the trend of the remainder of the data. These deviations are statistically significant, and the plotted points cannot be said to fall consistently about the best straight line drawn through them. Since there is no apparent break or curvature in the trend of the results but rather a wide variation of the plotted points about any single line that can be drawn, the conclusion is that there is a significant degree of heterogeneity in the animal responses. Such results are not infrequently encountered in dose-response investigations with drugs, and methods have been devised (5, 6, 7) for their statistical analysis. Whether the present results are characteristic of benzpyrene or whether they are fortuitous and are to be expected with methylcholanthrene and dibenzanthracene as well cannot be said at the present time. It is possible, however, that larger numbers of animals in the various dose groups would yield more consistent percentage responses.

The heterogeneity of the benzpyrene data makes it impossible to draw definite conclusions regarding the true nature of the dose response curve for this material and renders useless certain of the analyses that were made of the other hydrocarbons. However, other useful information may be obtained from the benzpyrene results, and the relation between dose and response may be determined within broad limits of error.

The curved lines of figure 23 show the limits of error of the calculated regression line corresponding to $P=0.05$. They were determined by methods applicable to the analysis of heterogeneous data (7). The equation to the regression line is:

$$Y=4.971+1.753\ (X+1.013) \qquad (9)$$

where Y is the calculated probit value and X the dose in log milligrams.

The percentage equivalents of the probit results are shown in figure 24. As judged from the S-shaped percentage curve without considering the limits of possible error, the useful dose range for benzpyrene is from about 0.012 mg. (TD_5) to about 0.875 mg. (TD_{95}).

Median tumor dose (TD_{50}).—Although subject to relatively large errors of estimate, TD_{50} can be determined within known limits. By appropriate procedures (7) log TD_{50} is found to be -0.996 ± 0.16. The limits of error corresponding to $P=0.05$ are $\pm$ 0.37. Thus, TD_{50} is

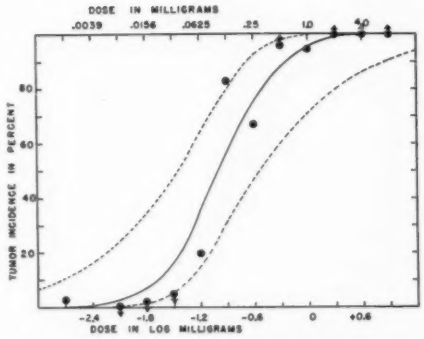

FIGURE 24.—Dose response curve for benzpyrene showing relation of tumor incidence in percent to log dose. Transformed from data of figure 23.

$0.101^{+0.046}_{-0.032}$ mg., and the limits of error for P=0.05 are $^{+0.135}_{-0.058}$.

Joint Use of Latent-Period and Tumor-Incidence Data

Total number of injected mice required to give latent-period responses of equal weight at various dose levels.—The total number of mice that must be injected to give the desired number of tumors (table 8) will depend upon the expected incidence of tumors at each dose level. Although the curve of expected percentages in figure 24 cannot be considered as being very accurately established, it may be used as an approximation in the absence of better information for judging the numbers of animals to be used in future experiments involving the latent period type of response. The expected percentages are given in table 8, as well as the total numbers of mice required for observations of equal weight when 10 are used at the level of maximum weight.

Specific induction time.—The specific induction time for benzpyrene is 5.23 ± 0.59 months (days/30). The limits of error corresponding to P=0.05 are ± 1.51 months.

COMPARISON OF RESULTS OBTAINED WITH THE THREE HYDROCARBONS

Tumor Incidence

The probit-dose response curves for methylcholanthrene, dibenzanthracene, and benzpyrene are plotted in figure 25. The curves for methylcholanthrene and dibenzanthracene (disregarding in the latter instance the higher dose range within which response is not correlated with dose) are fairly close together on the common dose scale, and the slopes of the two curves are essentially parallel. The curve for benzpyrene, however, differs from the others in both position and slope. Analyses were made to determine whether these differences were significant or whether they could have occurred by chance under the condition of the experiment.

Comparison of tumor-incidence response curves.—Statistical analyses show that the differences in slope are not significant as compared with their errors of estimate. This fact indicates that variations in slope of the magnitudes observed could have been due to chance under present condi-

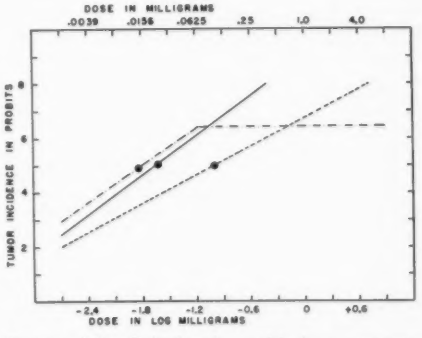

FIGURE 25.—Calculated probit-dose response curves for three carcinogenic hydrocarbons showing their relative positions on an absolute dose scale.
Solid line: Methylcholanthrene;
Dash line: Benzpyrene;
Dash-dot line: Dibenzanthracene;
Circles on lines: Means of experimental observations.

tions. Caution should be exercised, however, in considering the slope to be actually the same for the three materials since the errors of the estimates are relatively large, particularly in the instance of benzpyrene, and it is possible that larger numbers of observations might show significant differences not detectable under present conditions.

In spite of the wide range of error associated with the benzpyrene data, the response curve obtained with this material differs significantly, with respect to position, from those of the other two compounds which do not differ significantly from each other.

Estimation of the relative carcinogenic potencies of the three hydrocarbons.—The term "potency" as used here refers simply to the relative quantities of the various hydrocarbons required to produce a common biologic response. It is recognized that many factors would have to be considered in an attempt to estimate the absolute potency relationship, e. g., differential solubilities, relative rates of elimination from the body, relative rates of detoxification, etc. The potency ratios given represent, therefore, the apparent potencies of the respective hydrocarbons under the prescribed conditions of the experiment.

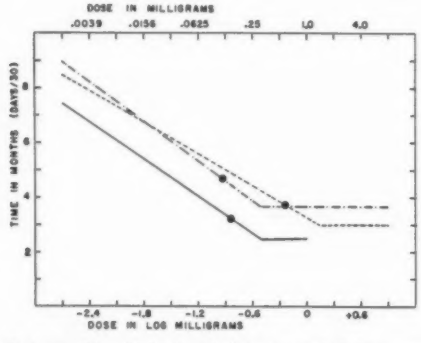

FIGURE 26.—Calculated latent period-dose response curves for three carcinogenic hydrocarbons showing their respective positions on an absolute dose scale.

Solid: Methylcholanthrene;
Dash: Benzpyrene;
Dash-dot: Dibenzanthracene.

The logarithms of the potency ratios were derived by obtaining the differences between the respective values for log TD_{50}. The antilogs of the differences represent the potency ratio, which is 6.3 : 4.8 : 1, in the order dibenzanthracene : methylcholanthrene : benzpyrene.[11] In terms of relative quantities required to produce the same biologic response (50-percent tumors), the ratio is 0.16 : 0.21 : 1. The difference between the relative potencies of methylcholanthrene and dibenzanthracene is not statistically significant, but both of these materials differ significantly from benzpyrene.

The magnitude of this difference in potency, however, cannot be stated with certainty in view of the wide limits of error associated with the results with benzpyrene. The values given represent the best estimates derived from present data. It has not been established that they represent exact values for the true potency ratios.

Latent Period

Comparison on an absolute dose scale (dose in milligrams).—The latent-period response curves for the three hydrocarbons are plotted in figure 26. The curves for methylcholanthrene and dibenzanthracene are essentially parallel, while that for benzpyrene has a somewhat flatter slope.

[11] *Shimkin* (11) *suggested the use of molecular weights rather than absolute weights for comparing relative potencies, "especially if the compared chemicals differ considerably in their molecular weights." The materials used in the present investigation do not differ appreciably in molecular weights. When their potencies are compared on such a basis, the ratio is 6.8: 5.2 : 1, in the order dibenzanthracene (molecular weight 268) : methylcholanthrene (molecular weight 268) : benzpyrene (molecular weight 252).*

Whereas the probit curves (fig. 25) for methylcholanthrene and dibenzanthracene are close together, the latent-period curves for these materials (fig. 26) are widely separated. The benzpyrene curve of figure 26 is nearest the dibenzanthracene curve which it crosses in the oblique, and again in the horizontal segment.

The horizontal portions of the curves in the range where response does not vary with dose are excluded from this and the following analysis. Tests of statistical significance show that the variations in slope are no greater than could have occurred due to chance alone under the conditions of the experiment. (See discussion of similar findings with respect to the probit curves on p. 524.)

The benzpyrene and dibenzanthracene response curves do not differ significantly from each other in relative position, but they both differ significantly from the curve for methylcholanthrene.

Estimation of the relative speeds of action of the three hydrocarbons.—It has already been pointed out (*1*) that latent-period responses are unsatisfactory for comparing the relative carcinogenic potencies of different hydrocarbons [12] since there is no point on the latent-period response curve which is definitely known to have a common significance for different compounds. Nevertheless, it is of value to know the relative speeds of action of different carcinogenic hydrocarbons when they are administered under similar conditions to similar test animals.

The curves of figure 26 indicate that on an average given quantities of dibenzanthracene and benzpyrene require considerably longer periods of time for the production of tumors than do similar quantities of methylcholanthrene. This is true when the comparison is made on a basis of equal quantities, e. g., milligrams, of the respective materials. Equal quantities of the hydrocarbons, however, did not have the same carcinogenic potency as measured by the tumor-incidence response; benzpyrene acted as if it were significantly less potent than the other materials.

If the latent-period data are considered alone, there is no point on the abscissa (dose) scale which could be chosen in preference to all others for comparing the relative speeds of action, i. e., the respective latent-period responses, and satisfactory comparison could be made only if the various response curves were parallel. Since the difference between the slopes was not found to be significant in the present instance, the curves were compared on a basis of a common slope (the compound slope). If the differences in slope should be found upon further experimentation to represent real differences, the present conclusions regarding speeds of action will not be strictly respresentative, since the relative speeds of action will vary at different levels of dosage.

The average differences between speeds of action when the materials were considered in terms of equal quantities were 1.22 months (days/30) for methylcholanthrene and dibenzanthracene, 1.86 months for methylcholanthrene and benzpyrene, and 0.64 months for dibenzanthracene and benzpyrene. The materials in order of rapidity of action were methylcholanthrene, dibenzanthracene, and benzpyrene. It is obvious from figure 26 that this order of relative speeds of action applies only to the oblique components of the respective curves.

Although the crossing of the oblique segments of the benzpyrene and dibenzanthracene curves could be fortuitous, there is little doubt that the horizontal segment of the dibenzanthracene curve crosses the

[12] *The latent-period response may, however, be used for determining relative potencies of different preparation of a given hydrocarbon.*

oblique segment of the benzpyrene curve since the horizontal segment of the latter is significantly lower than that of the former. The relative speeds of action of these two materials are therefore reversed when the higher doses are used.

In view of these findings, it would seem undesirable to make single dose comparisons between benzpyrene and dibenzanthracene with respect to the mean latent period. The comparison would have to be made over a wide range of doses since there is one dose, and possibly two, at which the mean latent period will be exactly the same for the two materials. At other doses the latent period for one material may be greater or less than for the other, depending upon the magnitude of the dose employed.

Comparison on a relative dose scale (dose in carcinogenic units).—Since the quantity of hydrocarbon composing one carcinogenic unit (*1*) was found to differ among the various materials, it would be of interest to know their relative rates of action when quantities possessing the same number of carcinogenic units are compared. Considering dose TD_{50} as one carcinogenic unit in each instance, the respective dose scales were converted to carcinogenic units by expressing the actual quantities of hydrocarbon as fractions or multiples of TD_{50}.

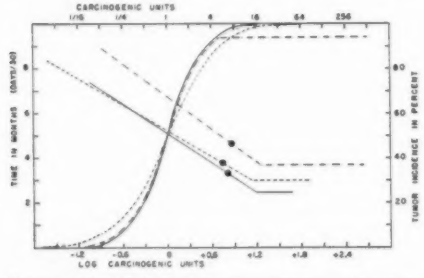

FIGURE 27.—Calculated response curves for three carcinogenic hydrocarbons showing incidence of tumors in percent and latent periods in months on a relative dose scale (carcinogenic units).
S-shaped curves: Incidence data;
Linear curves: Latent-period data;
Solid: Methylcholanthrene;
Dash: Benzpyrene;
Dash-dot: Dibenzanthracene.

The percentage and latent-period response curves for the three hydrocarbons are plotted in figure 27. The lower abscissa scale represents log carcinogenic units, the upper abscissa scale, the relative doses in carcinogenic units. It will be noted that the 50-percent points on the percentage response curves are superimposed at one carcinogenic unit. The respective points on the latent-period curves corresponding to this same dosage represent the specific induction times referred to.

When compared on a basis of similar tumor producing doses, the latent-period curve for benzpyrene falls much closer to the methylcholanthrene curve than to the one for dibenzanthracene (fig. 27). This is in contrast with the findings when absolute dosages were employed (fig. 26). The slopes of the respective response curves were not altered by conversion of the dosages to carcinogenic units. The positions were merely changed so that the respective values of TD_{50} coincided.

The analysis of slopes presented in the preceding section is applicable to the present series of curves. It may be reiterated, therefore, that the slopes were not shown to be significantly different.

When measured with respect to average position for each curve and on the basis of a common slope, the oblique components of the response curves for benzpyrene and methylcholanthrene do not differ significantly from each other. Both of these curves, however, differ significantly from that for dibenzanthracene. The horizontal components of the respective response curves differ significantly among them-

selves, that for benzpyrene being intermediate to the others in position.

Estimation of the relative speeds of action of equal carcinogenic units of the three hydrocarbons.—Comparison of the oblique segments of the curves of figure 27 with respect to their average positions and on the basis of a common slope indicates that tumors produced with dibenzanthracene appeared on an average 1.49 months later than with methylcholanthrene, and 1.22 months later than with benzpyrene. Tumors induced with benzpyrene appeared 0.27 months later than did those induced with methylcholanthrene.

With doses greater than 16 carcinogenic units (1.2 log carcinogenic units), the latent-period response of all materials was minimal. In the region of the minimal responses tumors were produced with dibenzanthracene 1.24 months later than with methylcholanthrene, and 0.7 months later than with benzpyrene. The tumors induced with benzpyrene appeared 0.54 months later than did those induced with methylcholanthrene.

If response curves are not parallel, the differences in respective speeds of action will vary at different levels of dosage, and as already pointed out it would be difficult to select a point of reference on the basis of latent-period data alone. In conjunction with the percentage data, however, it would be possible to compare relative speeds of action at doses giving a common percentage response and thus to have points of reference which possess a common significance in the instance of different hydrocarbons. The use of dose TD_{50} as a point of reference for comparing relative speeds of action has already been described (specific induction time (*1*)). If the present results are compared on a basis of their specific induction times, methylcholanthrene and benzpyrene differed by only 0.09 months in their respective speeds of action while the speed of action of dibenzanthracene was slower by 1.65 and 1.56 months, respectively, when compared with the other materials. These results are in contrast with the differences between minimal latent periods noted previously.

DISCUSSION

One of the primary purposes of this study was to supply additional data on the nature of the relationship between dose of subcutaneously injected carcinogenic hydrocarbons and the local tumor responses which they elicit in test mice. It is necessary to know the nature of the dose-response relationship in order that appropriate statistical procedures may be employed in analyses of the biologic results and in testing the significance of differences obtained under various experimental conditions. In a previous report (*1*) it was pointed out that statistical analysis of dose-response data is simplest when the relationship between dose and response is linear and that statistical methods are available for the analysis of data that follow this type of relationship. Attempts were made (*1*) to find appropriate units for expressing both dose and response so that the relationship between them would be linear. It was found that certain units which were applicable in the instance of most drugs were also applicable in the instance of carcinogenic hydrocarbons.

The findings of the previous study are substantiated by the present results. They are summarized as follows: (1) The mean latent period of tumors appearing in a group of mice, all of which have received the same dose of hydrocarbon, bears a linear relationship to the logarithm of the dose. This relationship is limited, however, owing to the fact that there is a minimal time below which tumors do not appear with further increases in dose. The minimal latent period appears to be

characteristic for a given hydrocarbon under constant experimental conditions. (2) The tumor-incidence responses, in percent, follow a symmetrical S-shaped curve possessing the characteristics of an integrated normal curve when plotted against logarithm of dose. Such S-shaped curves may be converted to a straight line by transforming the percentages to probability units (probits (*5*), or normal equivalent deviation (*12*)) and plotting them against the logarithm of dose.

The conclusions regarding the mean latent period-dose relationship are substantiated by the results with all three of the compounds employed. Additional evidence of a linear relationship between the mean latent period and the logarithm of dose was obtained by Gottschalk (*15*) in studies with benzpyrene.

The percentage-dose relationship was confirmed by the results obtained with methylcholanthrene and dibenzanthracene, but the data obtained with benzpyrene proved to be heterogeneous and not strictly definable by the relations of a normal curve. It is possible, however, that percentage responses to benzpyrene based on larger numbers of mice will give more homogeneous results. (See data of Leiter and Shear (*16*).)

A second objective of this study was to compare the relative carcinogenic potencies and the relative speeds of action of methylcholanthrene, dibenzanthracene, and benzpyrene. Attempts were not made to estimate absolute potencies but rather the relative actions, or the apparent potencies, of the three materials when administered under identical test conditions. For the conditions of the present experiments, the carcinogenic potencies were in the order dibenzanthracene, methylcholanthrene, benzpyrene; the ratio was 6.3:4.8:1. The potency difference between dibenzanthracene and methylcholanthrene was not statistically significant and the two may be exactly the same, or either may be slightly more potent than the other to a degree not detectable in the present experiment. In any event, it would not be surprising if the order were reversed in a subsequent experiment under the same conditions. There seems to be no doubt, however, that larger quantities of benzpyrene were required to produce a 50-percent response than of either dibenzanthracene or methylcholanthrene and that the apparent potency of benzpyrene is comparatively less under the conditions of these experiments. The order of potency here is the same as that found by Andervont and Shimkin (*17*) with regard to the induction of pulmonary tumors. Their results showed that dibenzanthracene produced the greatest number of pulmonary tumors, with methylcholanthrene next in potency. Benzpyrene produced only one-tenth as many pulmonary tumors as did methylcholanthrene and only one-sixteenth as many as dibenzanthracene when the materials were injected subcutaneously in equal quantities (*17*, *table 3*). This order of relative potencies of the three compounds is apparently contradictory to that usually given in the literature for the materials when subcutaneously injected. However, it should be pointed out that, to our knowledge, no previous comparative studies have been made in which the lower doses were used. Under the conditions of the present experiments doses of dibenzanthracene greater than about 0.1 mg. might be expected to give aberrant percentage results which, in the absence of a complete dose response curve, might be interpreted as indicating a lower carcinogenic potency for dibenzanthracene (fig. 25).

The most rapidly acting of the three compounds was methylcholanthrene, irrespective of whether comparisons were

made on an absolute dose basis (in terms of milligrams) or in terms of equal tumor-inducing doses (carcinogenic units). By using the latter comparison, the speeds of action were in the order methylcholanthrene, benzpyrene, dibenzanthracene. When comparisons were made on an absolute dose basis, the conclusions regarding the respective speeds of action of dibenzanthracene and benzpyrene depended on the dose level at which the comparisons were made.

In addition to the primary objectives of the experiments other useful information was obtained. For example, with all three hydrocarbons there was a significant correlation between the standard deviation of individual latent periods and the logarithm of dose. The range of variation of individual latent periods was strikingly large, especially in the region of the lower doses (figs. 1, 9, and 18). In the present experiments the various dose groups were made up by the addition of animals over a period of about 3 months, and it is possible that a wider range has been observed under these conditions than would have been obtained if the sampling had been made from a single lot of animals. The present results are probably more representative, however, of the variations to be expected in the population as a whole. The increasing range of variation of the latent period as the dose is decreased, coupled with the coincident progressive decrease in the percentage of mice with tumors, limits the usefulness of the latent-period response in quantitative studies to a very narrow dose range. For example, above the maximum effective dose, that which just produces the minimal latent period (*14*), there is no correlation between dose and response, and the latent period cannot be used as a criterion of quantitative biologic activity. On the other hand doses smaller than TD_{50} produce tumors in less than half of the injected mice, and the range of variation in the latent periods is so great that the numbers of mice required for reliable group responses are too large to be practical. The dose range within which the latent period might be effectively used is therefore from about dose TD_{50} to the maximum effective dose.

Another point of interest is the apparent common significance of the maximum effective dose of all three hydrocarbons. The maximum effective dose was defined by Fieser (*14*) as the dose that just produces the minimal latent-period response. Fieser noted that at this dosage the percentage response "approaches 100 percent." In previous work (*1*), the present authors questioned the use of the maximum effective dose for comparing the relative potencies of different carcinogenic agents because of the lack of proof that this criterion possessed the same significance, with respect to potency, for different agents. However, the present findings indicate that it does have a common significance, at least for the three hydrocarbons used. Reference to figure 27 reveals that the dose opposite the point of junction of the oblique and horizontal components of the latent-period response curve (the maximum effective dose) corresponds to the point on the corresponding percentage curve at which, for practical purposes, the latter just reaches 100 percent. Actually the approach to 100 percent is asymptotic, and the expression "just reaches 100 percent" is theoretically meaningless. However, as Foster (*18*) points out, the dose which gives a 99-percent response may for practical purposes be considered as that which just produces 100 percent.

The percentages corresponding to the maximum effective doses were calculated

from the probit regression equations to be 99.8 and 97.8 for methylcholanthrene, and benzpyrene, respectively. The dibenzanthracene percentage response curve did not continue to 100 percent (fig. 16), but the results became erratic with doses greater than 0.125 mg. If the trend of the responses at the lower doses is continued, however (see dotted extension of dibenzanthracene percentage curves of figures 16 and 27), the percentage response corresponding to the maximum effective dose is 99.8. The average percentage response corresponding to the break in the latent period curve is 99.1; the differences between the values for the various hydrocarbons are much less than the errors associated with the respective estimates. It is clear, then, that the dose which just produces the minimum latent-period response is also that which just produces the maximum percentage response and that the maximum effective dose (based on latent periods) may be considered as equivalent to about dose TD_{99}. For the three hydrocarbons under consideration, the maximum effective dose is approximately 16 times the dose that produces 50-percent tumors (TD_{50}).

Although the relationship between tumor-incidence response and maximum effective dose was essentially the same for the three materials in the present investigation, further study is necessary before the maximum effective dose (based on latent-period data alone) can be considered as a satisfactory criterion for the comparison of carcinogenic hydrocarbons in general. This criterion is definitely not applicable to another type of tumor-producing agent, the rabbit papilloma virus, of which the dose that just produces the minimal latent-period response is more than 16 times that which just gives the maximum (99-percent) tumor incidence (*19, fig. 8*).

SUMMARY

The quantitative relations between dose and tumor response are studied for the following carcinogenic hydrocarbons and dosages: 20-methylcholanthrene in serial twofold doses of from 0.00024 to 1.0 mg., and 1, 2, 5, 6-dibenzanthracene and 3, 4-benzpyrene both in serial twofold doses ranging from 0.00195 to 8.0 mg.

Both the tumor-incidence and latent-period types of response are analyzed in their relations to dose, by using appropriate biomathematical procedures.

The relative carcinogenic potencies and the relative speeds of action of the three materials are determined for the conditions of the experiment.

Useful relations between dose and response are presented in graphic form, and some implications of the various findings are discussed.

The computed values of certain of the most useful data are as follows (for limits of error of the various estimates, see text): Minimal latent period (average, in months (days/30)): dibenzanthracene 3.72, methcholanthrene 2.48, and benzpyrene 3.02. Median tumor dose (TD_{50})): dibenzanthracene 0.016 mg., methylcholanthrene 0.021 mg., and benzpyrene 0.101 mg. 95-Percent tumor dose (TD_{95}): dibenzanthracene 0.084 mg., methylcholanthrene 0.096 mg., and benzpyrene 08.75 mg. 5-Percent tumor dose (TD_5): dibenzanthracene 0.003 mg., methylcholanthrene 0.0045 mg., and benzpyrene 0.012 mg. Specific induction time in months (days/30): dibenzanthracene 6.79, methylcholanthrene 5.14, and benzpyrene 5.23.

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Wavelength Dependence of Tumor Induction by Ultraviolet Radiation

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If the effectiveness of various wavelengths in inducing tumors were accurately known so that an action spectrum could be reconstructed, it might be possible to identify the substance in the cell primarily affected by the radiation.[1] Unfortunately this is not feasible for a number of reasons, and one must be content with rather limited knowledge in this regard. The long wavelength limit for tumor induction by ultraviolet radiation has been set at approximately 3200A by several investigators ((*1*, *2*, *3*) and see (*4*)), and it has been shown that wavelength 2537A is little effective (*5*). The present studies add information regarding other wavelengths, and limiting factors which serve to mask the true action spectrum.

EXPERIMENTAL PROCEDURE

The only modification of the experimental procedure from that used in previous studies (*6*, *7*) was the interposition of glass filters, Corex D and Pyrex, between the source and the animals. The intensities of the lines of the mercury arc incident upon the animals when no filter is interposed and the transmission of the filters are given in table 1. From these data the intensities of the various wavelengths incident through the filters can be obtained and the exposure time required for a given dosage calculated. These data are given in table 2, where the time to 50-percent tumor incidence (t'_{50}) is also presented for groups receiving filtered radiation and for another group exposed without filter. Also included in this table are estimates of similar data for wavelength 2537A obtained by exposing mice to a low-pressure mercury arc (*5*).

In column 8 of table 2 are estimates of dosage that would induce tumors at the various values of t'_{50} if no filter were interposed. These were obtained by interpolation in the curve relating dosage and induction time published elsewhere (*8*). In column 9 the relative effectiveness for the various spectral regions is expressed as the ratio of the apparent effective dose (column 8) to the actual incident dose (column 5).

TABLE 1.—*Intensities of lines of the mercury arc and transmissions of filters and epidermis*

Wavelength	Incident intensity without filter ($\lambda<3200$A) (Ergs cm.$^{-2}$ sec.$^{-1}$ x 10^3)	Fraction of incident energy transmitted by—		
		Corex D filter	Pyrex filter	Irradiated epidermis [1]
3130A	13.2	0.76	0.40	0.28
3022A	6.8	.64	.12	.23
2967A	3.8	.52	.08	.17
2925A	.5	.45	.....	.14
2894A	1.2	.40	.....	.08
2804A	2.4	.17	.....	.02
2752A	.8	.10	.....	.02
2699A	.8	.05	.....	.02
2652A	4.8	.02	.....	.02
2602A	.2	.....	.....	.02
2537A	5.3	.....	.....	.02

[1] The method for obtaining these values is described by Kirby-Smith, Blum, and Grady (*9*).

Column 9 (table 2) indicates a wide difference in the effectiveness of different wavelengths. The maximum of effect

[1] *The action spectrum, which relates effectiveness to wavelength, should agree with the absorption spectrum of the light-absorbing substance under ideal conditions.*

must lie between 2600A and 3000A but cannot be much more accurately placed. The shorter wavelengths are little effective, as indicated by the low effectiveness of the 2537A line, and the fact that radiation passing through the Corex D filter, which cuts out virtually all wavelengths shorter than 2800A (see table 1), is relatively more effective than unfiltered radiation. The radiation passing through the Pyrex filter, which is composed principally of the 3022A and 3130A lines, is more effective than wavelength 2537A but less effective than the radiation passed by the Corex D filter, which contains wavelengths having the maximum effect.

TABLE 2.—*Spectral effectiveness in induction of tumors by ultraviolet radiation*

Group	Number of animals	Filter	Wavelengths	Weekly dose		t'_{50}	Weekly dose without filter estimated from t'_{50} (Ergs/cm.^{2} x 10^2)	Relative effectiveness at surface	Relative effectiveness below epidermis
				Incident on surface (Ergs/cm.^{2} x 10^2)	Incident below epidermis (Ergs/cm.^{2} x 10^2)				
						Days			
CH-CI	50	None	>2400A	8.8	1.3	167	8.9	1.00	6.75
CB	25	Pyrex	>2967A	8.0	2.1	300	0	.38	1.43
CA	25	Corex D	>2652A	4.1	1.0	187	6.5	1.59	6.50
BC	25	None	2537A [1]	36.0	.7	[2] 310	3.0	.08	4.30

[1] Low-pressure mercury arc used as source.
[2] Approximate value obtained by extrapolation.

In column 5 of table 1 are presented transmissions of the epidermis from the ear of a mouse which had been exposed repeatedly to ultraviolet radiation, and in column 6 of table 2 the amounts of radiation penetrating below the epidermis are estimated for the various groups using these transmission values as a basis. In table 2 the relative effectiveness of the radiation penetrating below the epidermis is estimated for the various wavelength regions. These values should represent more accurately the effectiveness of the radiation reaching the living cells than those of column 9 since epidermal absorption is principally that of the dead corneum (*9*).

The principal difference in the carcinogenic effectiveness of radiation incident on the cells as compared with that incident on the surface involves the shorter wavelengths. The effectiveness of wavelength 2537A is relatively much greater in the former case as is also that of the unfiltered radiation from which the wavelengths shorter than 2650A have not been removed. Obviously the filtering action of the corneum has a very important effect upon the spectral effectiveness of the radiation incident on the surface. The position of the maximum, however, appears to be unchanged so far as these measurements can determine. Unfortunately, this information still provides no clue as to the exact nature of the light-absorbing substances. These might be either nucleic acids whose maxima of absorption lie at about 2650A, or proteins which as a rule have their absorption maxima at about 2800A. The relatively high effectiveness of wavelengths 3000A or longer indicated by the effectiveness of the radiation filtered through Pyrex glass suggests the latter as the most likely, but this evidence is not at all conclusive.

There is a marked intensity dependence at low intensities (*10*). This could decrease the effectiveness of the radiation in the 2537A and Pyrex filter experiments. The extent of this effect is difficult to

estimate but is probably not an important factor and could not influence the conclusions drawn.

DISCUSSION

The hypothesis that the carcinogenic action of ultraviolet radiation results from induction of mutations in somatic cells has been entertained by various investigators. The action spectra for mutation production thus far obtained indicate that nucleic acid, rather than protein is the light absorber (*11*, *12*, *13*, *14*), and this corresponds to the belief that the nucleus is the site of action. Mackenzie and Muller (*15*) have suggested that the mutation hypothesis might be tested by determining whether the action spectrum for carcinogenesis corresponds to the absorption spectrum of nucleic acid. The present studies alone do not support or deny this hypothesis but may serve to point out the overwhelming task involved in obtaining sufficient data to decide the point. Determination of effectiveness for a single wavelength with any degree of accuracy demands the use of large numbers of animals, the numbers here used being insufficient for more exact studies (*16*). To expose this number of animals to monochromatic radiation would be outside the scope of any existing physical facilities. No existing monochromator would permit exposure of more than one or at most a very few animals at a time, and sources providing monochromatic radiation over a wide field, such as the low pressure 2537A mercury arc, have not been developed for other wavelengths in the spectral region to be studied. Not much better data than those presented in this paper could be obtained with filters even though much more extensive studies were made.

Even if an accurate action spectrum were obtained for radiation incident at the surface, it would have to be corrected for the absorption by the corneum in order to obtain the action spectrum for incidence at the level of the living cells, and this would involve an extensive study of the transmission of that layer, which would, in itself, be a major undertaking (*9*). Nor would the difficulty end at this point. We know how to compare action spectra and absorption spectra under certain conditions only. One condition is that absorption by the specific light absorber is a small fraction of the incident radiation (*17*). An alternative is that thorough stirring of the system is possible. Neither of these conditions exists in the present case, where absorption occurs in an indeterminately thick system (indeterminate because we do not know how to delimit the site of action accurately) composed of layers of inhomogeneous material. In spite of the difficulties an approximate action spectrum might be obtained which would be sufficient for the purpose, if the absorption maxima of the two suspected materials, protein and nucleic acid, were not so close together on the wavelength scale (*18*), but with the existing conditions the problem appears virtually insoluble. Thus it seems that testing of the hypothesis of somatic mutations must be accomplished by some other means.

Loss of or failure to gain body weight occurs in mice exposed to ultraviolet radiation. The magnitude of this effect parallels the amount of skin damage and may be considered a rough index of the destructive action of the radiation. The effect on body weight has been followed in the same experiments herein described, and the results have been presented in another communication (*19*). The two sets of measurements cannot be directly compared in terms of the spectral effectiveness of the radiation, because the relationship between dosage and effect is different in the two instances. Considering the

limitations of the accuracy of both sets of measurements, carcinogenic action and the effect on body weight cannot be said to have different action spectra. So far as these measurements are concerned, they may both result from the same primary photochemical reaction.

The action spectrum for the induction of tumors by ultraviolet radiation so far as it is described shows only that this phenomenon falls in a general class with many other types of action of ultraviolet radiation on living organisms, most of which are destructive in character. The action spectra of all these processes have approximately the same long wavelength limit and display maxima in the same region. It is probable that all these effects result from the action of the radiation on either protein or nucleic acid, but it is usually difficult, as in the present instance, to determine which type of compound is involved (*20*).

Thus no specific answer regarding mechanism is likely to be given by experiments of the present type. For example, both Mitchell's hypothesis (*21*) that split-products of protein resulting from the action of ultraviolet radiation act as carcinogens and the somatic-mutation hypothesis must receive equal credence if viewed only in terms of the data presented herein. On the other hand, these measurements make the assumption of a specific "wave band" for the carcinogenic process unnecessary. They also make it unlikely that carcinogenesis depends on the action of ultraviolet radiation on sterols, because of the nature of the absorption spectra of those compounds (*18*). Together with evidence previously presented (*5*), they indicate that the corneum is not involved in the carcinogenic process, and that the action of the radiation is on the living cells themselves or at least in their immediate neighborhood.

SUMMARY

Measurements of the effectiveness of different wavelengths in induction of tumors by ultraviolet radiation have been made using cut-off filters.

The importance of the screening action of the corneum in modifying the action spectrum is pointed out.

The substance primarily affected by the radiation is probably either protein or nucleic acid; but it is highly improbable that this type of experiment can decide the point, since physical and biologic limitations do not permit sufficient accuracy of measurement.

The bearing of these measurements on theories of carcinogenesis is discussed.

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Effect of Intensity on Tumor Induction by Ultraviolet Radiation

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In previous studies of induction of tumors by ultraviolet radiation (*1*, *2*, *3*), it was found that the intensity of the radiation had but slight effect on the tumor-induction process. An additional experiment, using a wider intensity range and a greater number of animals, was carried out to establish the magnitude of the intensity effect. The results are given herein.

EXPERIMENTAL PROCEDURE

The same technique was used in this experiment as in those previously described (*1*, *4*). Ninety-four strain A albino mice were used. All received the same dosage, 1.8×10^7 ergs per square centimeter 5 days per week. Half the animals (groups CO and CP) received this dosage at 3.3×10^4 ergs per square centimeter per second intensity which required approximately 9 minutes' exposure, whereas the other half (groups CQ and CR) received 1.8×10^3 ergs per square centimeter per second which required approximately 160 minutes' exposure. The ratio of intensities was thus about 18 to 1. The time to 50-percent incidence of tumors, t'_{50} (*2*), was 164 days for the high-intensity group, and 212 days for the low-intensity group. On the basis of statistical studies previously presented (*2*), such a difference in t'_{50} would be expected to occur due to chance alone less frequently than once in 100 times when groups of 44 animals are compared. Thus, the results are highly significant, and the existence of an intensity effect seems established by this experiment alone. Comparison of these results with those previously obtained permits further conclusions. The basic data for all experiments in which intensity was varied are assembled in table 1.

TABLE 1—*Basic data on effect of intensity of radiation on tumor development time*

Groups	Animals alive or with tumor at t'_{50}	Dose[1] (Ergs per cm.^{2} per exposure x 10^7)	Intensity[2] (Ergs/cm.^{2}/sec. x 10^4)	t'_{50}	Apparent effective dose[3] (Ergs per cm.^{2} per exposure x 10^7)
	Number			*Days*	
F	24	3.3	3.4	142	3.3
B	25	3.3	1.3	154	3.3
G	24	2.0	3.4	148	2.0
C	26	2.0	.8	145	2.0
CO–CP	44	1.8	3.3	164	1.70
CQ–CR	44	1.8	.18	212	1.08
AI	25	1.6	1.6	164	1.70
AE	23	1.6	.80	186	1.35
AA	23	1.6	.50	161	1.80
AJ	25	1.1	1.6	206	1.14
AF	24	1.1	.80	211	1.10
AB	25	1.1	.34	213	1.10
H	24	.76	3.4	287	.68
D	24	.76	.30	347	.52
AK	21	.72	1.6	250	.84
AG	23	.72	.80	277	.72
AC	21	.72	.23	336	.54

[1] All groups received 5 exposures per week.
[2] The intensities presented in previous papers (*1*, *2*) are in error due to miscalculation. The correct values are 80 percent of the values there presented. The values in column 4 are correct. This error in previous papers does not affect any of the conclusions presented therein.
[3] Values were obtained by interpolation of t'_{50} on the dose response curve (*3*, *fig. 1*) for 5 exposures per week.

INTERPRETATION OF RESULTS

In table 2 groups are compared which received the same dose of radiation, but at different intensities. The groups are arranged in pairs for comparison, each pair being given a number to aid in discussion (column 1). The pairs are arranged in the order of the lowest intensity received by either group (column 3).

In column 4 are recorded the differences between t'_{50} for the low-intensity group and t'_{50} for the high-intensity group. The dif-

TABLE 2.— *Effect of intensity on induction of tumors by ultraviolet radiation*

Comparison pair	Groups ($\frac{\text{high intensity}}{\text{low intensity}}$)	Lowest intensity for pair (Ergs/cm.^{2}/sec. x 10^4)	Difference in t'_{50} (percent of high intensity value)	Probability of difference in t'_{50} due to chance alone	Ratio of intensities ($\frac{\text{high}}{\text{low}}$)
1	F/B	1.3	+8	0.2	2.6
2	G/C	.8	−2	.7	4.2
3	AI/AE	.8	+14	.05	2.0
4	AJ/AF	.8	+2	.7	2.0
5	AK/AG	.8	+11	.2	2.0
6	AE/AA	.5	−15	.02	1.6
7	AI/AA	.5	−2	.7	3.2
8	AJ/AB	.34	+3	.5	4.7
9	AF/AB	.34	+1	.9	2.3
10	H/D	.30	+21	.01	11.3
11	AK/AC	.23	+34	.01	7.0
12	AG/AC	.23	+21	.01	3.5
13	CO–CP/CQ–CR	.18	+29	.01	18.3

ferences are expressed in percents of t'_{50} for the high-intensity group. If t'_{50} is greater for the low-intensity group, a positive sign is given; if t'_{50} is greater for the high-intensity group, a negative sign is given. In the majority of cases the sign for this value is positive, indicating that the appearance of tumors is slower on the whole in the groups exposed to the lower intensities. The probability that this difference in t'_{50} might occur due to chance alone has been calculated for each pair. In doing this, the pooled standard deviation value 0.084 log days, obtained from previous data, has been used. These probabilities are presented in column 6. The differences for pairs 3, 6, 10, 11, 12, and 13 are statistically significant, but there is reason to believe that the apparent significance of pairs 3 and 6 should be disregarded. Both these pairs include the group AE which seems to have an inordinately long t'_{50} which may be considered as due to chance when one takes into account the total number of groups which have been studied, i. e., it is the only group in over 40 that exceeds the accepted limits of statistical significance when compared with similarly treated groups, and the probability of 0.02 for pairs 3 and 6 indicates a difference of this magnitude is to be expected once in 50 times due to chance alone. This group is one of three (AA, AE, and AI) which received the same dose, the intensities increasing in the order named (table 1). The difference in t'_{50} for the extremes (pair 7) is not significant, and since t'_{50} for AE is greater than for either of the extreme groups the differences for pairs 3 and 6 (column 4) have opposite signs. This does not fit any reasonable interpretation when the other data are considered. Thus there seems every reason for disregarding the apparent significance of pairs 3 and 6.

Only pairs 10, 11, 12, and 13 display highly significant differences of t'_{50}, i. e., these differences would occur only once in 100 times due to chance alone. All have positive signs. The greatest ranges in intensity are found among these pairs, as may be seen by reference to the ratios in table 2, column 6, and the greatest difference in t'_{50} might be expected in these cases. However, in some instances greater ranges of intensity did not produce significant differences, e. g., compare pairs 2, 8, and 12. Pairs 10–13 include the four lowest intensity groups, and this suggests that the intensity dependence is greater at lower intensities. In fact, it appears that there is little or no increase in t'_{50} until the intensity is decreased below 3.4

x 10^3 ergs per square centimeter per second, and this becomes more clearly evident when the data are analyzed quantitatively.

While comparison of t'_{50} serves for statistical interpretation and qualitative demonstration of an intensity dependence, quantitative comparisons should be made in terms of effective doses. The curve relating dosage and t'_{50} has been established and was presented in an earlier paper (*3*). The apparent effective dose for any group can be estimated by interpolation of its t'_{50} on this curve. This has been done, and the values are given in table 1, column 6. The ratios of the apparent effective dose to the true dose have been plotted against the intensity in figure 1. It appears from this figure that no significant reduction is brought about by reducing the intensity from 3.4 x 10^4 to about 4 x 10^3 ergs per square centimeter per second, but that reduction below the latter value brings about a definite decrease in effectiveness. The break is obviously sharp although it need not be as abrupt as is indicated in the figure. The conclusion may be drawn that above about 4 x 10^3 ergs per square centimeter per second the effectiveness of ultraviolet radiation in inducing tumors is virtually independent of intensity, but that below this approximate limit effectiveness falls off rapidly with intensity.

The lack of important interdependence between dose and intensity is indicated by a comparison of columns 3, 4, and 6 of table 1.

DISCUSSION

These observations point out a potential source of error in such experiments. Wide variation in intensity of the radiation will not affect the results so long as it does not fall below a certain critical value; but if the intensity is allowed to fall below this

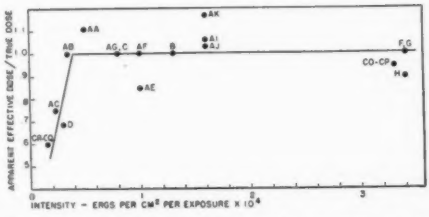

FIGURE 1.—Influence of intensity on the effectiveness of ultraviolet radiation in inducing tumors.

limit, variations in induction time must occur which may lead to serious misinterpretations. For our particular experimental conditions, i. e., male strain A mice exposed to a particular type of intermediate pressure mercury arc (*1*), this lower limit is about 4 x 10^3 ergs per square centimeter per second. However, this is not an absolute value; for, if another type of source were used having a different spectral distribution of its emitted energy, this lower limit would be different (*1*). Moreover, if the same source were used with animals with a different epidermal transmission, the lower limit would be different (*5*). Thus the critical intensity should be established for any given set of experimental conditions.

It may be pointed out that in all our experiments the intensity has been maintained within close enough limits to establish the accuracy of the results. In considering dosage relationships (*3*), experiments in which the intensity was purposely maintained at a low level were eliminated, a procedure which is justified by the present analysis. Reproducibility of experimental results can be high, as shown by our series of experiments (*2*, *3*), but only if dosage and intensity are taken into consideration and estimated with appropriate accuracy.

These experiments show that the reciprocity law holds down to a certain intensity, below which there is a sharp

deviation from this relationship. This suggests that if the changes brought about by ultraviolet radiation do not take place at a sufficiently rapid rate, the cells are able to recover from them; and thus at lower intensities of radiation the number of cells altered falls off, and tumor induction time is increased. Above a given rate of change the cell is unable to recover sufficiently; hence the induction time is not altered by increasing the intensity. The phenomenon observed by Rentschler, Nagy, and Mouromseff (*6*) on the killing of bacteria by ultraviolet radiation is not observed here. These investigators found that prolonged exposures at low intensities were more effective than short intense exposures when the same amount of energy was given in both cases. This they explain by the assumption that the cell is more susceptible at certain periods of its cycle, and that the cells are more likely to be encountered in susceptible stages if the exposure is prolonged. Since in the present instance the cells were intermittently exposed for a large number of times, it is not surprising that such an effect was not observed.

The demonstration of intensity dependence at low intensities is of particular importance with regard to the question of the role of the ultraviolet radiation of sunlight in the induction of human cancer. As has been pointed out elsewhere (*5*, *7*), the intensity of the tumor-inducing wavelengths (shorter than 3200A) of sunlight is much lower than that of a mercury arc of the type used in these experiments. It is probable, therefore, that the intensity dependence is an important limiting factor in induction of tumors by sunlight. This would help to explain the low incidence of cutaneous tumors in man, relative to experimentally induced tumors in mice, a matter which has been discussed elsewhere with regard to total dosage and to susceptibility (*5*, *7*, *8*). Not only is the intensity of the carcinogenic wavelengths of sunlight much less than that of the mercury arc, but for equal incident intensity the intensity at the level of the viable cells is much less in man than in the mouse because of the greater absorption by the human stratum corneum (*5*).

The fact that not only the total amount of radiation of wavelengths shorter than 3200A must be considered but also the intensity, should increase the dependence on geographical latitude for induction of tumors by sunlight. At higher latitudes the intensity must be diminished on account of the greater zenith angle and the correspondingly greater mass of air through which the sun's rays must pass (*7*). Unfortunately we have no way of estimating the quantitative relationships for the dependence of intensity on latitude unless an extrapolation is made from our observation on mice, and this cannot be expected to have great accuracy. Consequently, intensity must be recognized as a factor when attempt is made to correlate cutaneous cancer incidence in man with latitude, but great hope for a successful quantitative evaluation cannot be entertained.

SUMMARY

Tumor induction in male strain A mice by ultraviolet radiation is virtually independent of intensity above a certain critical intensity. Below this critical intensity carcinogenic effectiveness falls off sharply.

The importance of controlling intensity in experimentation is discussed and also the importance of this factor in interpreting the role of sunlight in human cutaneous cancer.

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Neoplasms and Other Lesions of the Eye Induced by Ultraviolet Radiation in Strain A Mice

By STUART W. LIPPINCOTT, *formerly passed assistant surgeon (R),*[1] *and* HAROLD F. BLUM, *senior pharmacologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

A study of the lesions induced in the eyes of strain A mice by ultraviolet radiation is reported herein and extends observations by Grady, Blum, and Kirby-Smith (*1*), who reported the occurrence of sarcomas and hemangio-endotheliomas arising from the cornea of such animals when they were exposed repeatedly to this agent. Attention is focused, in the present paper, upon the changes which may accompany or precede the development of neoplasms. Much of the material for histopathologic studies was obtained in the course of investigations of induction of cutaneous tumors. Tumors of the eye were obtained only from those mice that developed cutaneous tumors or from those dying before they developed such tumors. Under these conditions, about 5 percent of the animals showed gross tumors of the eye. More would undoubtedly have been observed if the mice had received further exposure after the appearance of the cutaneous tumors. A much larger number of the animals developed lesser lesions of the eye, which in most instances manifested themselves grossly as opacities. Probably few, if any, of the eyes of the exposed animals were entirely normal.

The technique of exposure of the animals to the radiation is described elsewhere (*2*).

HISTOPATHOLOGY

The whole eye, together with the eyelids, was dissected from the orbit. The tissues were fixed in dilute formalin 1:10 U. S. P. for approximately 1 month and then sectioned by the paraffin method. The haematoxylin and eosin stain was used routinely; phosphotungstic acid-haematoxylin stain was employed in some preparations.

LESIONS OF THE CORNEA

Various components of the cornea are susceptible to the effects of ultraviolet radiation. The pathologic changes occasionally affect only one layer but more frequently involve two or more layers. The lesions may be widespread or occur as focal alterations. The lesions show degenerative, inflammatory, and neoplastic changes in varying combinations.

Epithelium

Normally, the epithelium in this strain of mouse is a layer 4 to 5 cells thick (fig 1, *A*). The cells are flattened in the outer portion and cuboidal or ovoid in the innermost layer. Generalized epithelial hyperplasia resulting from exposure to ultraviolet radiation is seen in figure 1, *B*. In the regions of epithelial hyperplasia, the outer layer shows evidence of parakeratosis while the inner layer is composed of globular, irregularly arranged cells, which vary considerably in size. Frequently, spongiosis may be identified. The cells are vesicular, and their nuclei contain 2 or more nucleoli. The average cellular depth

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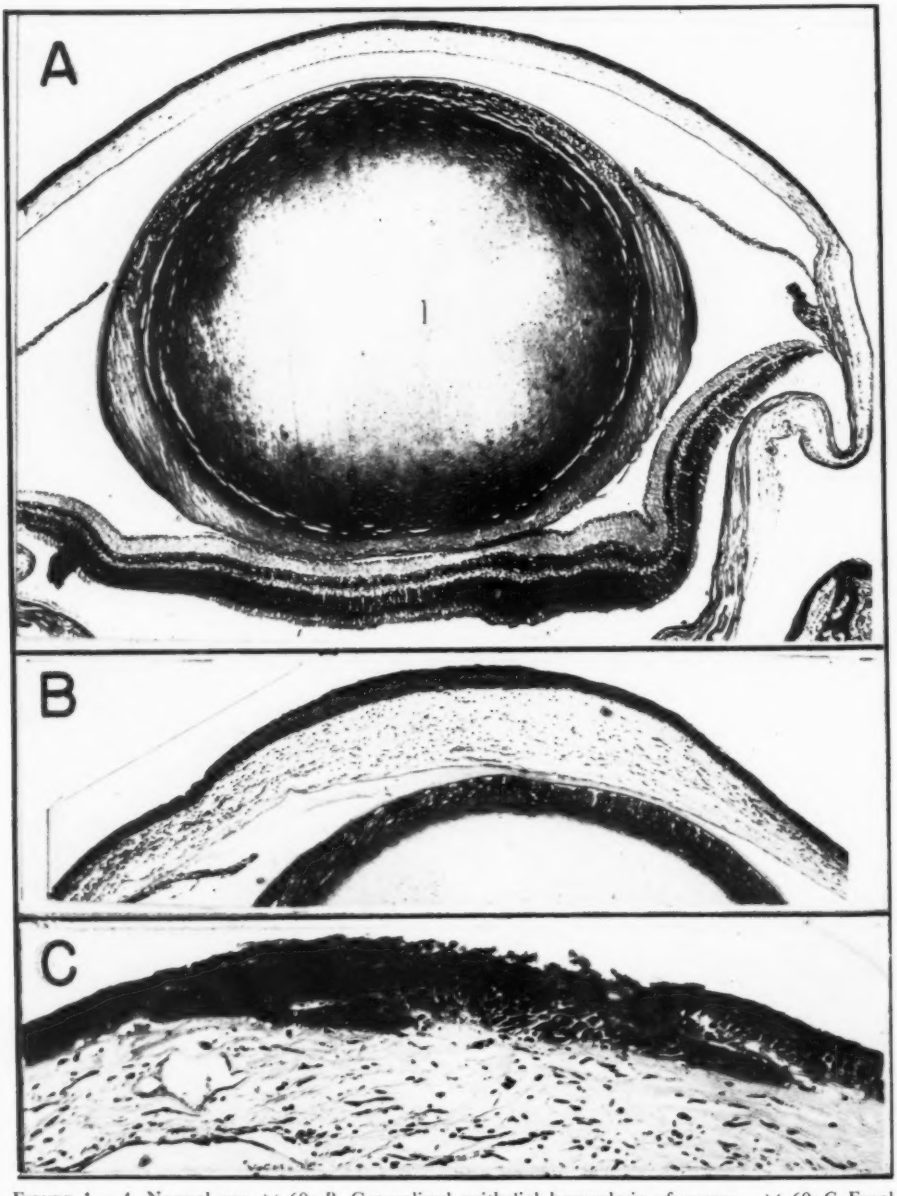

FIGURE 1.—*A*, Normal eye. × 60; *B*, Generalized epithelial hyperplasia of cornea. × 60; *C*, Focal epithelial hyperplasia of cornea. × 165. Haematoxylin and eosin stain used throughout.

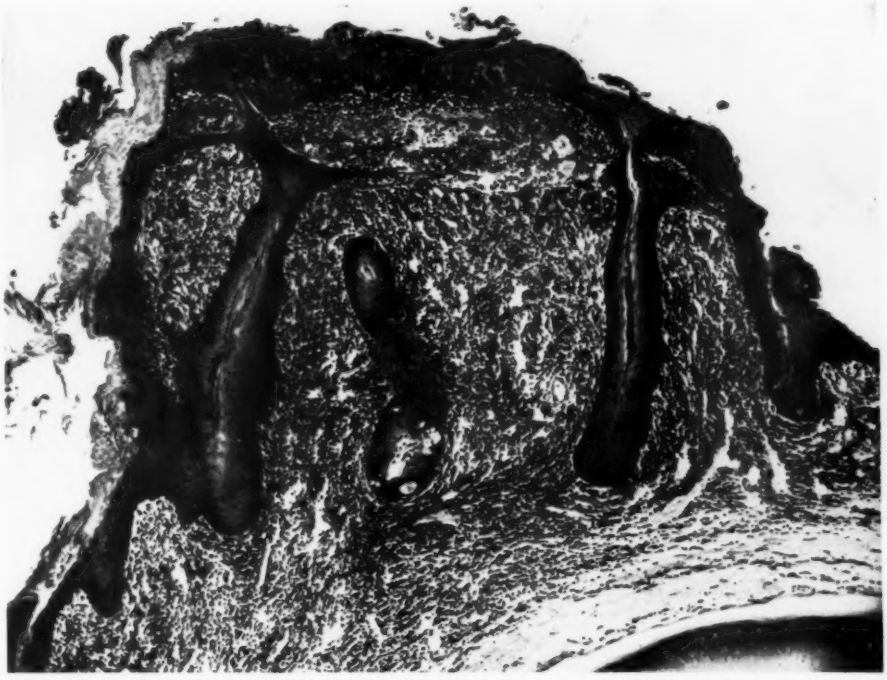

FIGURE 2.—Extensive epithelial hyperplasia approaching the category of an early squamous carcinoma, together with extensive inflammatory reaction. Haematoxylin and eosin stain. × 80.

in the most hyperplastic areas is about 12 cells. Hyperchromatic nuclei are not seen; mitotic figures are absent; and Bowman's membrane remains intact. The greatest amount of cellular proliferation appears centrally and tapers out towards the lateral portions of the cornea.

Focal epithelial hyperplasia usually occurs in the central portion of the cornea and is striking when present (fig. 1, *C*). The appearance of the cells is similar to that in generalized epithelial hyperplasia, but in addition there are distinct extension of the epithelial cells into the underlying substantia propria and loss of Bowman's membrane. Rarely, this process becomes very pronounced, as shown in figure 2. Here there are epithelial downgrowths in which the basal cells appear somewhat hyperchromatic and in which as many as five mitotic figures may be seen in a single high-power field. The basement membrane is no longer preserved at some points. There is, in addition, an extensive inflammatory process, and in the stroma are many spindle-shaped connective tissue cells. Occasionally a cell is plumper than its fellows, but is otherwise typical in appearance. While the lesion illustrated in figure 2 has been classified as epithelial hyperplasia, it seems to approach early squamous-cell carcinoma.

Substantia Propria

The substantia propria may display generalized or focal vascularization. The generalized change is seen in figure 3, *A*. The substantia propria appears irregularly

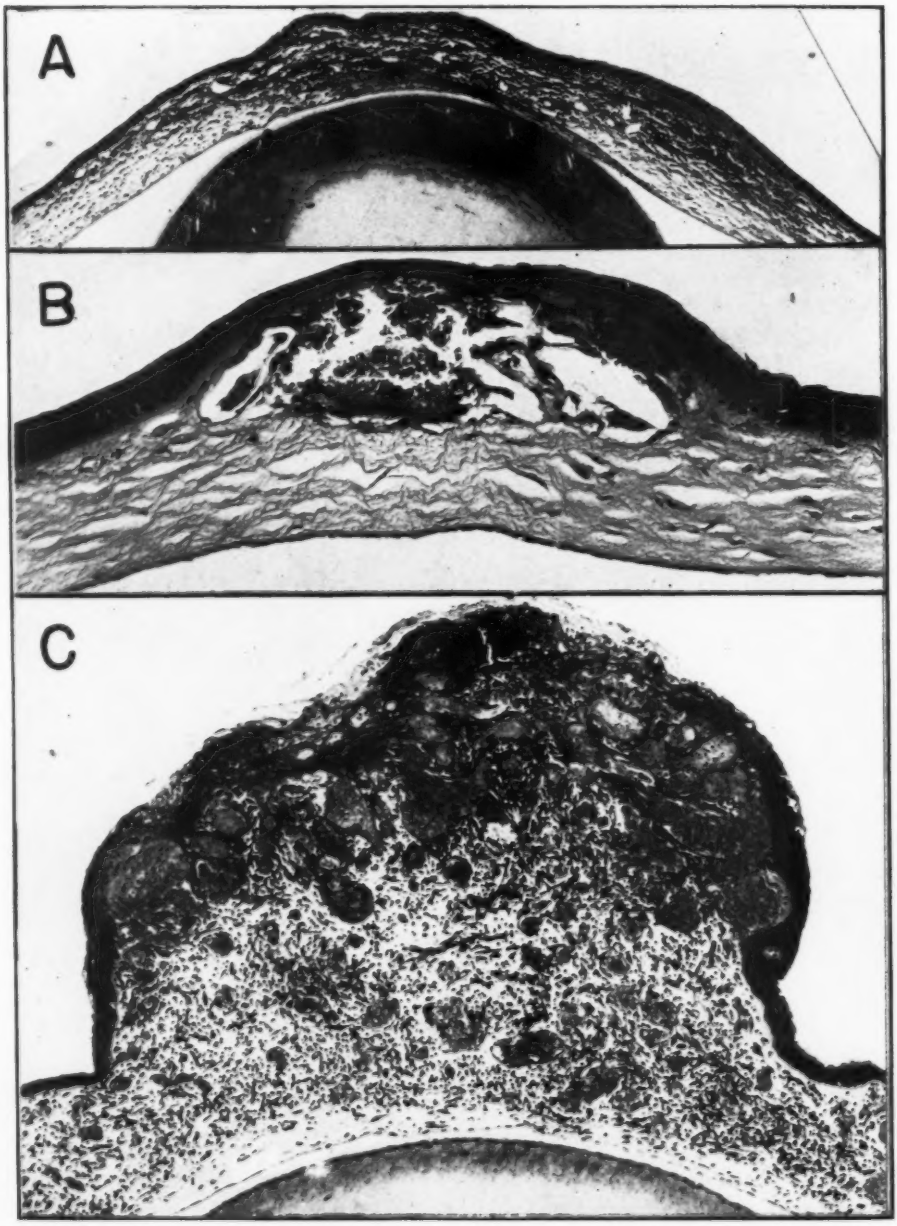

FIGURE 3.—*A*, General vascularization in substantia propria. × 60; *B*, Localized vascular reaction in substantia propria. × 220; *C*, Hemangio-endothelioma. × 215. Haematoxylin and eosin stain used for all.

thickened and contains within it many slender, elongated capillaries, each lined with a single layer of flattened endothelium. There is also a proliferation of stellate and spindle-shaped connective tissue cells interwoven among the vascular channels. Some small, round, deep-blue-staining cells resembling lymphocytes are scattered throughout the areas of denser vascularization. The endothelium and Descemet's membrane appear intact. Again, the greatest amount of alteration is in the central portion. There is less change as the lesion fans out laterally.

Localized vascularization usually appears in the anterior portion of the cornea as shown in figure 3, *B*. In this type of lesion the newly formed vessels appear as sinusoids, together with communicating smaller capillaries. Many red blood cells are in the lumens. The endothelial lining is orderly in arrangement. Neoplastic transformation of similar vascular channels may occur (fig. 3, *C*). Here, there is a destruction of overlying epithelium, with some irregular proliferation and fairly abundant desquamation of epithelial cells. Inflammatory exudate is also present. The neoplasm is composed of numerous small and large vascular channels lined with endothelial cells, usually with hyperchromatic nuclei, which are plumper than the cells seen in the simple vascularization. These endothelial cells sometimes project out from the channels into the stroma as single cells rather than sheets of cells. In the stroma there are also stellate and spindle-shaped connective tissue cells, regular in arrangement, together with numerous leukocytes and some pigment resembling hemosiderin. Such lesions have been considered to be hemangioendotheliomas.

The majority of neoplasms are fibrosarcomas occurring in the substantia propria and infiltrating other portions of the eye. One of these is illustrated in figure 4. The neoplasm in this instance is composed of interlacing bundles of spindle-shaped cells which are generally uniform

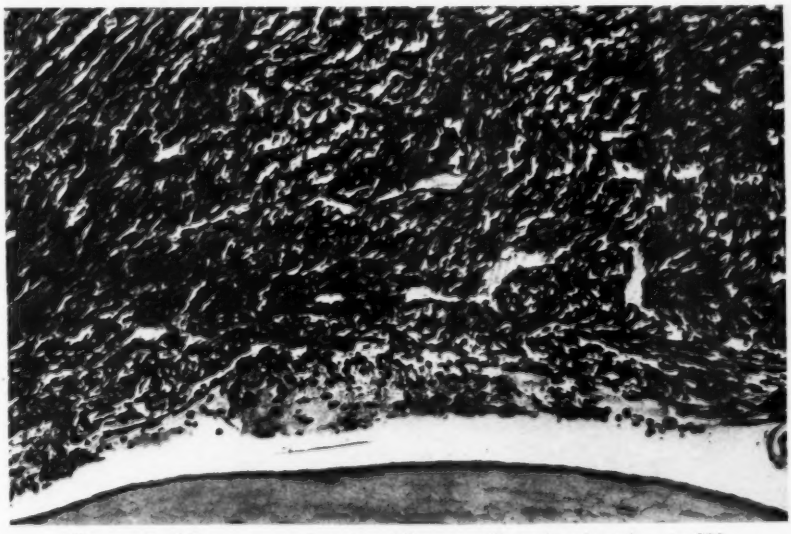

FIGURE 4.—Fibrosarcoma of cornea. Haematoxylin and eosin stain. × 200.

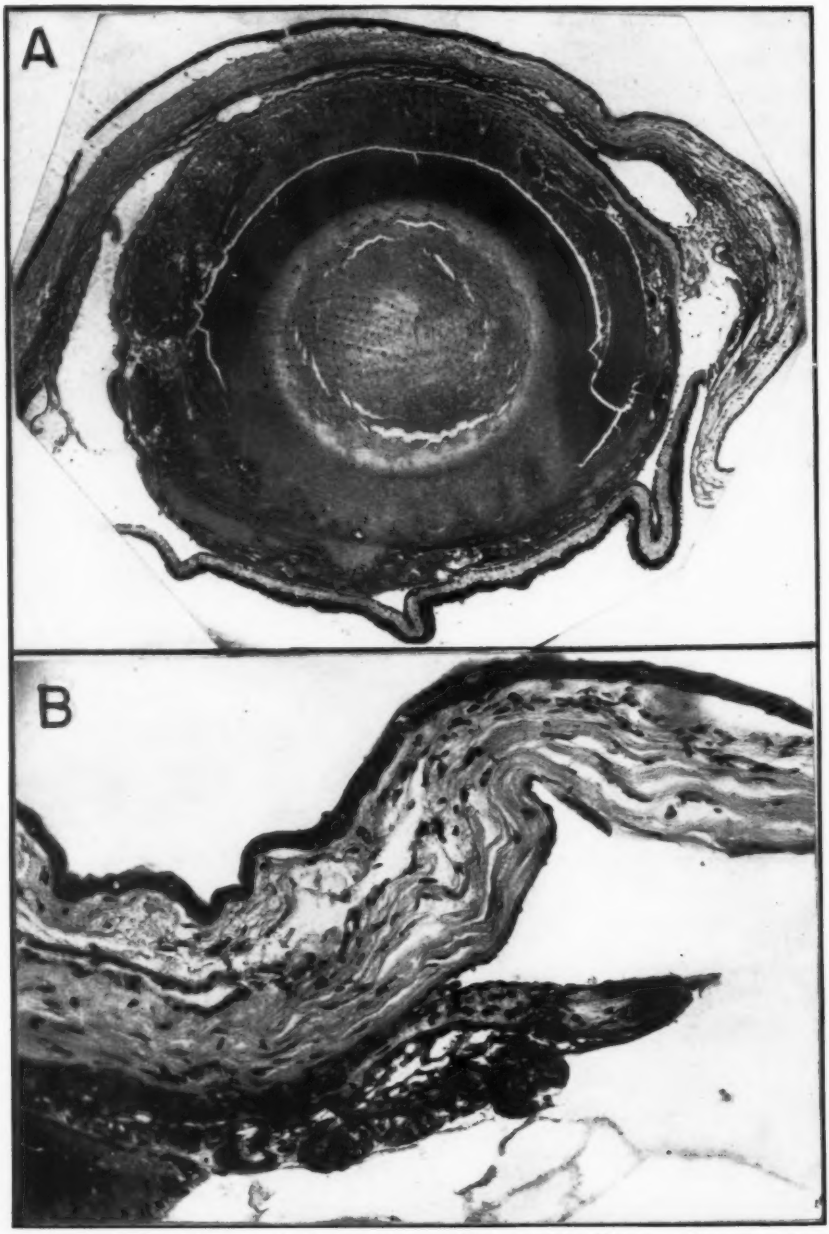

FIGURE 5.—*A*, Iridocyclitis. × 60; *B*, Anterior synechia. × 260. Haematoxylin and eosin stain used in both *A* and *B*.

in appearance. They are hyperchromatic. As many as six mitotic figures may be seen per high-power field. Occasionally, an atypical, rather large, spindle-shaped cell is interspersed. There is no palisading. Slender vascular channels lie between the compact bundles of connective tissue cells. The individual groups of cells appear anaplastic, and there is active infiltration. Fibroglia fibrils may be identified in sections stained with phosphotungstic acid.

Lesions of the Iris

When there is a lesion in the cornea, there may be an associated lesion in the iris. Definite iridocyclitis is evident in figure 5, *A*. In this section the iris on one side is fused with the substantia propria. A generalized inflammatory process makes it difficult to distinguish the iris as an individual structure. A less pronounced involvement of the iris is seen in figure 5, *B*, in which there is an anterior synechia.

Lens Changes

In a few instances histopathologic changes were observed in the lens itself which were diagnosed as cataract. These appeared to be secondary to other lesions, and there is no evidence that cataract had been produced by the primary action of ultraviolet radiation. Technically, it is difficult to prepare sections of an eye as small as that of the mouse in which all the eye structures are preserved intact. In most sections of normal eyes, the central portion of the lens appears to be paler staining and more homogeneous. Occasionally, there is a cracking of the lens substance which is artifact and is not to be confused with true cataract. This is shown in figure 1, *A*. Some of the cataracts observed appear to have resulted from fusion of the cornea with the lens. Such a lesion is shown in figure 5, *A*. The lens substance is irregularly broken up, and there are distinct differences in the staining quality of the fragmented portions of this region of the lens. Scattered throughout this part are single and occasionally small groups of what appear to be epithelial cells. True Morgagni's globules have not been identified. There is complete destruction of the normal lenticular fibrillation. No giant cells are evident, but deposits of calcium salts are seen (fig. 6, *A*). In contrast, there may be complete fusion of the anterolateral portion of the lens with the cornea, without transformation of the lens into a cataract (fig. 6, *B*). It seems probable that secondary cataracts appear only when there has been extensive and prolonged adhesion between cornea and lens.

DISCUSSION

As might be expected, the majority of the lesions produced by ultraviolet radiation are confined to the more superficial structure of the eye, since the destructive wavelengths shorter than 3200A do not penetrate very deeply. The principal site of action is the cornea where most of this radiation must be absorbed, and involvement of other structures seems to be secondary to changes in this region.

Both the epithelium and substantia propria of the cornea display lesions. Numerous fibrosarcomas have been found; but no definite carcinomas have been diagnosed, although lesions of the epithelium which suggest early malignancy have been observed. This parallels the preponderance of sarcomas over carcinomas among tumors of the skin induced by this agent, clear-cut difference in response of the epidermal and connective tissues being demonstrated in that case (*1*).

Vascularization of the cornea is not a specific response to ultraviolet radiation.

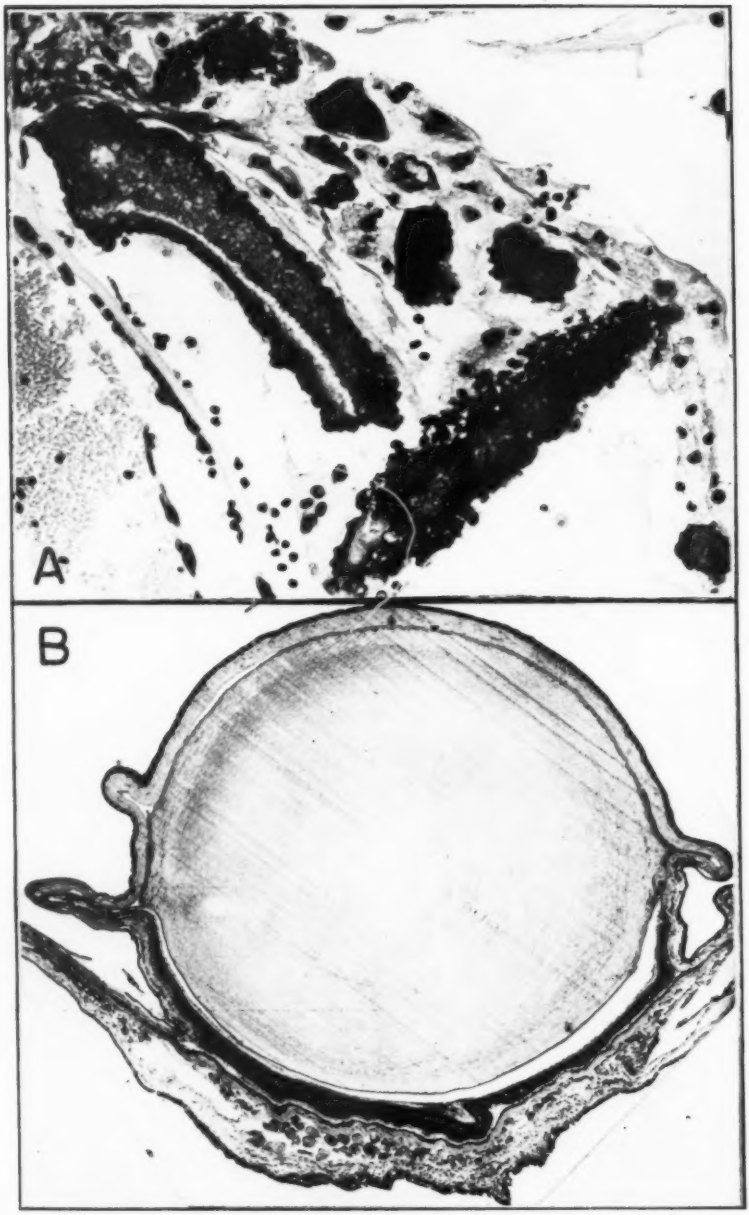

FIGURE 6.—*A*, Calcium salt deposits. × 260; *B*, Fusion of lens to cornea without formation of cataract. × 60. Haematoxylin and eosin stain used in both *A* and *B*.

In the vitamin-A-deficient rat (*3*), and in the riboflavin-deficient rat (*3*) and mouse (*4*), extensive growth of vessels from the limbus is observed. Bessey and Wolbach (*3*) suggest that the vascularization results from local asphyxia in the case of these vitamin deficiencies, but it seems improbable that this is the specific cause in the present instance. Products resulting from the destruction of cell constituents, including histaminelike substance, are usually formed when tissues are exposed to ultraviolet radiation, and these may provide the stimulus to vascularization in this case, rather than asphyxia. As the result of continued exposures to ultraviolet radiation, all stages of transition from single focal nonneoplastic vascularization to true hemangio-endothelioma may be observed.

In a recent report, Ash and Wilder (*5*) state that the incidence of tumors of the limbus of the eye is greater in the Southern States, although they feel it necessary to qualify this statement because of a possible selection factor in their material. The incidence of cutaneous cancer is also high in these States; and since this seems best accounted for as owing to the carcinogenic action of the ultraviolet radiation in sunlight, insolation being high in these regions (*6*), it seems reasonable to suggest that tumors of the limbus may also be induced by this agent. The fact that tumors of the cornea can be induced experimentally by ultraviolet radiation lends support to this hypothesis. The relative incidence of tumor types is quite different in the two cases, however. Among tumors of the eye experimentally induced in mice by ultraviolet radiation, sarcoma is the predominant type, paralleling the findings for experimentally induced cutaneous tumors. This is in contrast with the case with human beings, where carcinomas predominate, both among tumors of the skin and of the limbus of the eye. This discrepancy has been explained for cutaneous tumors on the basis of the depth of penetration of the radiation, and differences in response of epithelial and connective tissues (*1*, *7*). The same factors may be active in the case of the eye. The occurrence of hemangio-endotheliomas in mice is another point of difference since such tumors are not reported for the human eye. However, their relatively high incidence in mice parallels the readily induced vascularization of the cornea in the mouse.

It is of interest that the lens is not primarily affected. No direct measurements of the penetration of ultraviolet radiation to the lens of the eye in the mouse are available, but comparison of this organ with mouse epidermis on which we have such measurements suggests that wavelengths shorter than 3200A may reach the lens in considerable amount. The stratum corneum of the epidermis of the skin probably absorbs the major part of the incident radiation because of its scattering properties which enhance its absorption (*7*), yet in spite of the presence of this layer a considerable fraction of the incident radiation may reach the corium. Scattering at the surface of the cornea cannot be comparable in degree with that which takes place in the corneum; and although absorption by the cornea and aqueous humor must be considerable, it is probable that an appreciable amount of radiation reaches the lens. However, cataract is not induced. In the human eye the penetration of these wavelengths to the lens must be much less since the thickness of the absorbing layers is much greater. Hence the hypothesis that ultraviolet radiation is an important factor in cataract in man must be open to question.

The fact that a variety of lesions of rodent eyes may result from exposure to

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ultraviolet radiation suggests that the action of sunlight on the human cornea and conjunctiva should be more carefully studied. In addition to malignant changes, visual disturbances need consideration (*8*), as well as the possibility that lesions resulting from this source might be confused with those accompanying deficiency diseases (*9*).

SUMMARY

Neoplasms and other lesions of the eye induced by ultraviolet radiation are described. Pathologic changes are superficial, being confined primarily to the cornea. Hyperplasia of the epithelial and connective tissue elements, and vascularization are observed. There may be inflammatory changes, and iris and lens may be involved secondarily.

The tumors observed have been sarcomas and hemangio-endotheliomas of the substantia propria. Changes in the epithelium of the cornea suggest that carcinomas may occur at times.

The possible etiologic role of sunlight in producing lesions of the human eye is discussed.

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Changes Induced in a Strain of Fibroblasts From a Strain C3H Mouse by the Action of 20-Methylcholanthrene

(Preliminary Report)

By W. R. EARLE, *senior cytologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

Earle and Voegtlin (*1*, *2*) demonstrated that when rat and mouse fibroblasts were grown *in vitro* in an entirely heterologous culture medium consisting of a mixture of chicken plasma, horse serum, and chick-embryo extract and containing various concentrations of 20-methylcholanthrene, the cells underwent a transformation which did not reverse when they were removed from the carcinogen. The general character of these altered cells was remarkably similar to that of the cells of cultures of sarcoma produced by the injection of methylcholanthrene into the subcutaneous connective tissues of the rat.

Because of circumstances mentioned in the second of these papers, it was not possible to test exhaustively whether the inoculation into rats or mice of the altered cells produced by the action *in vitro* of the carcinogen was capable of producing malignant tumors.

In the present work, these studies were extended with four general aims. These were:

(1) To confirm the previously observed cell changes with a more accurate control of the concentration of methylcholanthrene.

(2) To follow with greater accuracy the progression of changes in the cells, both in the carcinogen and after removal from it, and in particular to follow the sequence of changes during the first 60 days after exposure to the carcinogen (this interval corresponding approximately to the latent interval for production of tumors by injection of the carcinogen *in vivo*), and to observe in greater detail the later cell changes, particularly those occurring after cells are subjected to the carcinogen for more than 250 days.

(3) To attempt to demonstrate conclusively a change from the normal to the malignant cell by the action of the carcinogen *in vitro*. This of necessity involved the production of tumors by injections of cells treated with the carcinogen *in vitro*.

(4) To correlate the relative tumor-producing ability of the cells with morphologic changes observed in them.

Because of necessary delay in the publication of a detailed report on this series of studies, this preliminary report is made on the cell changes brought about and the production of tumors from the injection of the carcinogen-treated cultures. In the preliminary report immediately following, the pathology of the tumors produced is outlined by Dr. A. Nettleship (*3*), who carried out the pathologic studies.

A primary strain of fibroblasts from the subcutaneous and adipose tissue of a 100-day-old male mouse of the C3H strain (Andervont substrain) was started *in vitro* in horse serum, chick-embryo extract, and saline solution on October 18, 1940. Cultures were grown in Carrel D3.4

flasks. When the strain reached an age of 291 days *in vitro*, the first addition of purified methylcholanthrene was made to selected groups of the cultures in the form of a solution of such strength that final concentration of the carcinogen in the fluid culture medium was 1γ per cubic centimeter.

Selected groups of the primary strain of cultures were carried in the carcinogen for different intervals; each group had its own untreated control. Particular attention is directed to groups or cell strains designated H, J, L, N, and O, which were subjected to the carcinogen for 6, 32, 111, 184, and 406 days, respectively, at the end of which time they were carried on without further addition of carcinogen. Until January 1, 1943, these strains were carried 391, 391, 391, 512, and 512 days, respectively, since the first addition of the carcinogen, and 385, 359, 280, 328, and 106 days since they were removed from the carcinogen.

The first noticeable effect of the carcinogen was a definite depression in the rate of increase in width of the cultures. This was noticeable after a very few days in the carcinogen and in its preliminary stages at least seemed to arise from a retardation of cell proliferation. This depression in the rate of increase of size of the cell clump has continued ever since, even after the cells were removed from methylcholanthrene. It appeared that the apparent retardation of the rate of cell proliferation was also present at about 400 days in the carcinogen, but whether the depression of the rate of increase in width of the cell clump arose from this factor alone was not determined. The conclusion was reached that the action of the carcinogen on the cells was progressive and gradual, and in the course of time almost certainly lethal.

The earliest recognizable morphologic change in the cultures appeared at about 40 days after first exposure to the carcinogen. In general, the cells showed a gradual diminution in the length of the terminal processes. The lateral edges of the cell processes became increasingly amoeboid in appearance, and this change extended progressively from the tips of the cell processes toward the center of the cell as the exposure of the cells to the carcinogen was lengthened. The cells gradually tended to send out numerous lateral processes at their free edges; meanwhile, the cells became increasingly coherent, particularly laterally, with the production first of cell strands and cell ribbons and finally of sheets which showed many characteristics of epithelial sheets in tissue culture. As time went on, these sheets became increasingly massive, while the cell cytoplasm became extremely granular.

The cell changes induced in different cell strains by different intervals of exposure continued after further addition of carcinogen to the cultures was discontinued. To date, two cell strains subjected to the carcinogen in the concentration mentioned for 6 and 32 days, respectively, have been carried approximately 1 year since the carcinogen was discontinued and without loss of their induced cell characteristics. Other strains have not been carried so long without carcinogen, but they also show no diminution of the changes induced.

Over 8 months after the first recognizable morphologic cell alterations in the earliest group of experimental cultures, and approximately 2 months after the last carcinogen-treated strain (strain H) had shown recognizable cell alterations, the control cultures started to show definite alterations of the same type as the experimental cultures, but to a lesser degree. Of the various control cultures examined, those of strain H showed least

alteration, the change in architecture of the cultures of this strain being barely discernible morphologically. The possibility of whether this change arose from a spontaneous tendency of the apparently normal fibroblast to undergo a malignant change under cultivation *in vitro*, or whether it arose from a trace contamination of the control cultures with carcinogen will be discussed in the detailed report. Although the data available do not allow a final conclusion, it is thought likely that such trace contamination with carcinogen did occur; however, the way in which it might have occurred is unknown. In view of the precautions taken to guard against contamination, it is emphasized that the contamination was probably extremely slight. Until further data are available to define more clearly the active concentration of the carcinogen necessary to induce changes in cells in long-term tissue cultures, great care should be taken in the handling of such cultures with carcinogen to eliminate any possibility of complications that might arise from trace contamination.

After all deliberate treatment of the cells with carcinogen had been discontinued and after strains H and J had been carried nearly 1 year from discontinuance of the carcinogen while other strains had been carried for shorter periods, the characteristics of the control cultures and the cultures which were subjected to the carcinogen for 6, 32, 111, 184, and 406 days were compared. The progression of morphologic changes noted was similar to that described.

The similarity of the latent period for production *in vivo* of the tumor with methylcholanthrene and the time necessary for the production of recognizable alteration of the cell *in vitro* is pointed out.

When control cultures or when carcinogen-treated cultures of strains H, J, L, N, and O were injected into young adult C3H mice at various intervals following removal from the carcinogen, tumors arose at the sites of injection. The time required for tumors to appear and their relative rates of growth varied somewhat from series to series, but the time to first appearance of an easily palpable tumor that continued to increase in size was often as short as 9 days, and the tumors regularly increased to such an extent that they caused the death of the mice within a very few weeks. The growth of tumors from treated cultures of strains N and O was somewhat slower and more erratic. Tumors produced from each strain of cells were successfully subinoculated, usually without difficulty. Tumors from strain L treated cultures produced metastases to the lungs in a number of cases. Of a total of 545 primary inoculations of mice from strains H and D control cultures and from strains H, J, L, N, and O carcinogen-treated cultures, a group of 378 injections was selected as being made under approximately comparable conditions. These represent strain H controls, other control strains, and strains H, J, L, N, and O carcinogen-treated cultures. The number of inoculations for each of the strains and the percentage of tumors produced are given in table 1.

TABLE 1.—*Percentage of tumors produced in relation to degree of culture change*

Culture strain	Number of injections	Tumors produced	Degree of culture change
		Percent	
H control	39	7.7	Barely perceptible.
D and other controls.	83	86.0	Greater than for H control.
H treated	65	72.0	Greater than for D and other controls.
J treated	45	20.0	Greater than for H treated.
L treated	54	46.0	Greater than for J treated.
N treated	66	7.6	Greater than for L treated.
O treated	26	7.6	Most changed of any.

The conclusion was drawn that the percentage of tumors produced by injections of cultures morphologically altered by the action of the carcinogen started at a very low value (7.7 percent) for the H strain controls, barely perceptibly altered morphologically. The percentage then rose to 86 for D strain controls, which were definitely altered, probably reached a maximum of nearly 100 just a little short of treatment with carcinogen for 6 days, and then dropped rapidly with progressive morphologic cell alteration, reaching a low of 7.6 with the morphologically altered cells of strains N and O, subjected to the carcinogen for 184 and 406 days, respectively. The extreme morphologic alteration of strain O *in vitro* and the low tumor incidence obtained from such cultures indicate that the treatment of these cultures had gone far beyond the optimum conditions for the production of tumors.

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Morphology of Sarcomas Derived From Fibroblasts Previously Treated With 20-Methylcholanthrene in Vitro

(Preliminary Report)

By ANDERSON NETTLESHIP, *passed assistant surgeon (R), National Cancer Institute, National Institute of Health, United States Public Health Service*

This report gives a brief description of the gross and microscopic characteristics of tumors which resulted from the inoculation into C3H mice, of fibroblast cultures described in the preceding paper (*1*). It includes tumors of strains D, H, J, L, N, and O, both primary and the transplants derived from each strain.

GROSS PATHOLOGY

Ordinarily the tumor presented itself at the site of inoculation as an irregular, globular, hard mass, firmly attached to the muscles and skeleton. All primary tumors resulting from the inoculation of tissue cultures into mice were hard; the transplant tumors, particularly in later transplants, were often very soft. The tumors averaged 18 x 20 x 25 mm. in size. On section, in the smaller primary tumors there was scant necrosis, but in the larger tumors and in later transplants there was considerable. The type of necrosis was commonly central, spreading in an irregular, fan-shaped fashion into the outer zone of firm pink tumor which was spared. Scattered hemorrhages appeared along the outer edge of the necrotic zone. The tumor edge which had grown into muscle could not be defined. The free border was rarely adherent to the skin and showed no capsule. A few tumors which had progressed to large size invaded the skin and ulcerated through the surface.

If the implantation had been deep, the tumor grew into the back or thigh muscles, through them, and into and about the left kidney or pelvis. In either case, the pelvic and abdominal organs showed widespread implantation. The tumor grew among the coils of the small intestine, in the mesentery and omentum, among the lobes of the pancreas, and into the capsule of the spleen. It was difficult to distinguish which of these tumors occurred by direct extension and which were metastatic. Frank metastases were found in the lungs and mediastinum. Those in the lungs were situated deep in the parenchyma and beneath the pleura, and appeared similar to the abdominal masses. In those cases with the most extensive involvement, emaciation was extreme and death commonly resulted. Splenomegaly of unexplained origin was an almost constant finding.

HISTOPATHOLOGY

The histopathology of all these tumors was that of the classic spindle-cell sarcoma of mice, as described by Haaland (*2*), Slye, Holmes, and Wells (*3*), and Heidenhain (*4*). Although there was much variation between strains, the sarcoma was very similar to the large and small spindle-cell sarcomas encountered in man. The majority of the tumors were of the large spindle-cell type; in some transplants the

small spindle-cell variety was common. The cells encountered in the large-cell variety were characteristically of the long fibroblast type, with light, granular, eosinophilic cytoplasm. The nuclei were large and contained scant karyoplasm and one or two nucleoli of moderate size. The cells were in broad sheets or in interlaced bundles. The commonest variation from the interlacing pattern of cells was fasciculation. Fibroglial fibers were found, and silver impregnation showed reticulum about the individual sarcoma cell. In many areas the stroma was well-developed.

The sarcoma cells invaded the surrounding normal structures by direct extension and pushed between the parenchyma cells of whatever organ was attacked. Nerves were found which were almost destroyed; blood vessels which had been invaded were thrombosed. In the skin the sarcoma cells destroyed collagen and elastic fibers. Bone was destroyed by involvement of the periosteum and destruction of the cortex, and finally the bone-marrow cavity was invaded. Muscle and kidney were destroyed by invasion between individual units. The structures of the pancreas were spread apart and destroyed.

No metastases to lymph nodes were found although one node was involved by direct extension. Metastases which were found in the lung did not differ from the original tumor, except for the lack of necrosis. The metastases appeared to be of vascular origin since blood vessels in the lung were found filled with tumor emboli. The sarcoma cells grew out from the vessels in a circular fashion, compressing and destroying the surrounding alveoli. In the cases in which metastases were noted, the tumor of origin was highly necrotic. Necrosis in all of these sarcomas was extremely variable. Some areas of necrosis were fresh, showing cells in early phases of destruction, while other areas contained macrophages filled with hemosiderin. In these latter areas granulation tissue was abundant. Little fresh hemorrhage was seen although the extensive granulation tissue gave the appearance of such in the gross.

The different strains were compared microscopically from the standpoint of the variability of their cell pattern, the capacity of the cells to invade, the number of mitoses, the number of giant cells, the occurrence of metastases, and the amount of necrosis. This gave a rather complete picture of the malignant properties of each tumor strain.

The D strain and other control strains which were originally carried as controls compared closely with the H and J strains. Of all strains studied, the D strain had the most constant and even histologic pattern. There was invasion of muscles, mitoses were moderate, necrosis was minimal, and no giant cells or metastases were found.

In the H and J strains the number of mitoses was about the same as in the D strain. Invasion was present, but the sarcoma cells tended to grow about structures, rather than into them as in the case of the L strain. Necrosis was moderate. A few scattered giant cells were found, but no enlarged nuclei or unusual mitotic figures. No metastases were found. The cell pattern was a little more variable than that in the D strain.

The L strain showed a more malignant alteration of the sarcoma cells. The cells invaded markedly and indiscriminately; metastases occurred most often in this strain; mitoses were abundant. Necrosis was more marked than in the previously mentioned strains.

In the N strain the cell pattern was even more variable. Large nucleated giant

cells began to appear, necrosis was variable, and no metastases were found. Many of these tumors were of the small-cell variety. Mitoses were diminished in comparison with those in previous strains.

The O strain showed a radical departure in architecture from all other strains. It presented an unusual picture, with many large nucleated giant cells, aberrant and bizarre mitoses, and the greatest variability in cell pattern. Although the tumor invaded, metastases were found only in a single instance. In this case the metastases were multiple.

These characteristics of the various sarcoma strains are summarized in table 1.

TABLE 1.—*Morphologic characteristics of various sarcoma strains*

Sarcoma strain	Exposure time	Irregularity of cell pattern	Invasiveness	Mitoses	Giant cells	Metastases
	Days					
D and other controls	.....	+	+	+	.....	.....
H treated	6	+	+	+	.....	.....
J treated	32	+	+	++	+	.....
L treated	111	++	++	+++	+	+++
N treated	184	+++	++	++	+	.....
O treated	406	++++	++	+	+++	+

These observations, furthermore, have an important bearing on the classification of sarcomas. Although all these sarcomas arose from tissue cultures which were originally derived from connective tissue, the morphology of the tumors suggested many varieties of sarcomas, such as neurogenic, angiomatous, myxomatous, and round- and giant-cell types.

The histologic pattern of these sarcomas became more irregular in proportion to the length of time the original tissue culture had been in contact with the carcinogen. This was closely connected with the ability of the sarcoma to invade and metastasize (table 1). An optimal exposure time for malignancy (strain L) was found, in which invasion and metastases were at a maximum. Below this optimal time the sarcoma structure was regular, mitoses were moderate, and invasion was minimal. No metastases were found. When a longer exposure time was used, the sarcoma showed extreme irregularity of structure, with many large nucleated giant cells, low mitotic rate, and rare metastases.

We may conclude from these observations that a low degree of exposure of the culture to the carcinogen produced cells of a low degree of malignancy, with a slightly distorted cell structure. With a higher degree of exposure, the cells became most malignant, without great distortion of their architecture. With a still higher exposure, not only was the cell structure greatly changed but also the actual invasive and metastasizing properties of the malignant cells were lowered.

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Study of a Spontaneous Mouse Rhabdomyosarcoma

By Anderson Nettleship, *passed assistant surgeon (R), National Cancer Institute, National Institute of Health, United States Public Health Service*

A previously normal 3-month-old female strain C (Andervont) mouse was found, on May 20, 1942, with a mass on the lateral surface of the right thigh. The tumor was solid, attached firmly to the underlying structures, but had not grown into the skin which was freely movable in this region. On incision through the skin, a hard, homogeneous, gray tumor, 15 x 14 x 9 mm., was found. This tumor lay at the junction of the thigh and pelvis and from its under surface had grown directly into both structures. It bulged outward in a thick, rounded, glistening globe. On section it was gray-pink and somewhat nodular, closely resembling cut muscle in color and consistency, with no definite lower border. The gross aspect of the tumor in transplant is shown in figure 1, *A*. Some small hemorrhages were seen but no cysts. The most striking aspect of the tumor was its extreme hardness—it felt like muscle in contraction. Although there was no certain diagnosis, trocar transplantation was immediately effected into a number of normal strain C mice. Gross examination revealed no other lesions. There was a slight thymus enlargement.

The histologic section showed a wild array of smaller than normal, musclelike, invasive, neoplastic cells; in some areas these cells were invading the surrounding fat, normal muscle, and pelvic bone. A number of large channels contained blood. There were also bone and cartilage in the tumor which may or may not have belonged to the pelvis proper. The tumor contained more cells of an adult type than in previously reported human cases, and the degree of polymorphism was not so extensive. The usual definitive characteristics, such as large, elongated, and spindle-shaped cells with sharply outlined cytoplasm, elongated and oval nuclei with abundant chromatin, cells grouped together in bundles or in fasciculated groups, were present (fig. 1, *B*). When compared with normal muscle cells the tumor cells were found to be much smaller. Many of the cells contained complete cross striations with all normal components present, anisotropic and isotropic disks, including Z lines (fig. 2, *A* and *B*). Cross sections in such cells showed regularly distributed myofibrillae. There were other varieties of cells present: scattered giant cells with few nuclei; small, round cells with dark-stained nucleus and little cytoplasm; larger, oblong or polygonal cells with poorly stained cytoplasm and clear nuclei with single or double nucleoli (fig. 2, *B*). Other cells of unusual shapes were seen. Mitoses were frequent. Special stains yielded results consistent with the haematoxylin-and-eosin section just described. Mallory's aniline blue stained the tumor cells a variable purple and the surrounding connective tissue blue. Phosphotungstic acid-haematoxylin stained the tumor cells a bright purple-red bringing out the cross striations prominently. With the Verhoeff-van Gieson stain the tumor cell cytoplasm was light yellow, the cell nuclei black, and the connective tissue pink. Reticulum stain and Foot's silver impreg-

nation showed a fine reticulum which had individuated almost every tumor cell but was not so heavy as that of normal muscle (fig. 3, *A*). Stains for nerve endings (Bodian's) failed to reveal any endings on the sarcoma cells. Careful examination gave the impression that the tumor cells were largely in contraction, and this fits in well with gross examination in which the firmness of the tumor was noted. Other interesting aspects of the tumor were the large blood channels with simple endothelial linings and the bone and cartilage. It was impossible to be certain that these latter elements belonged solely to the tumor or to the pelvis. Other abnormal elements were not found. No metastases were found, and all other organs were histologically normal.

In spite of the large number of mature cells in the tumor which contained definite cross striations there were still many cells which probably represent stages in the process of differentiation (*1*). The variability in these types of cells is shown in (fig. 3, *B*). This particular tumor had little of the embryonal character of the more common rhabdomyoblastoma. The most distinctive characteristic of the stroma of these tumors has been described as precollagenous fibers around the individual cells (*1*). These fibers were seen in this tumor. The most distinctive feature of the tumor cells themselves, the presence of fibrils, was well shown, and the type of fibrils with such well-defined cross striations marked this tumor as being of a more adult type than any previously described. That these adult cross-striated cells were not normal muscle is clear from their distinctive structure. Careful examination of the cross striations led to the belief that many of the cells were in contraction.

Under transplantation two elements have persisted, the rhabdomyosarcoma and the blood channels. Küttner and Landois (*2*) reported similar channels. Richmond (*3*) mentioned cystic areas. The rhabdomyosarcoma reproduces itself faithfully, and the blood channels now form large blood-filled cysts, which are very prominent. In spite of efforts to rid the tumor of the cysts by transplanting only the solid portion, the cysts, one to three or four in number, appear in each transplant. The takes during the first three generations were very few, but at present they are over 50 percent. No metastases have as yet been seen.

When the tumor was first observed, since no cysts were seen grossly, it was considered to be a pure rhabdomyosarcoma arising in a young animal. However, prominence of the cysts on transplantation caused a reconsideration. The following possibilities were thought of: teratoma with predominant muscle and blood-vessel elements and small deposits of bone and cartilage, Wilm's tumor, and endothelioma. The lack of glandular elements and the placement of the tumor, even though it was not far from the urogenital ridge, rather ruled against Wilm's tumor. The predominance of muscle was against endothelioma. We were left then with a tumor largely rhabdomyosarcomatous in nature. Study of this was revealing, be-

FIGURE 1.—*A*, Drawing of gross specimen of tumor, *in situ* and sectioned, on the posterior surface of the left thigh. First intramuscular transplant, growth about 6 weeks. The large, blood-filled cysts rest on the superior surface and are distinctive on cut surface. The solid portion of the tumor is gray-white. *B*, Drawing of microscopic field showing some polymorphism. Note irregular arrangement of the cells and the scattered, small, dark, round cells, also the cross striation. From original tumor. Haematoxylin and eosin stain. Approximately × 600.

A.

B.

INEZ DEMONET

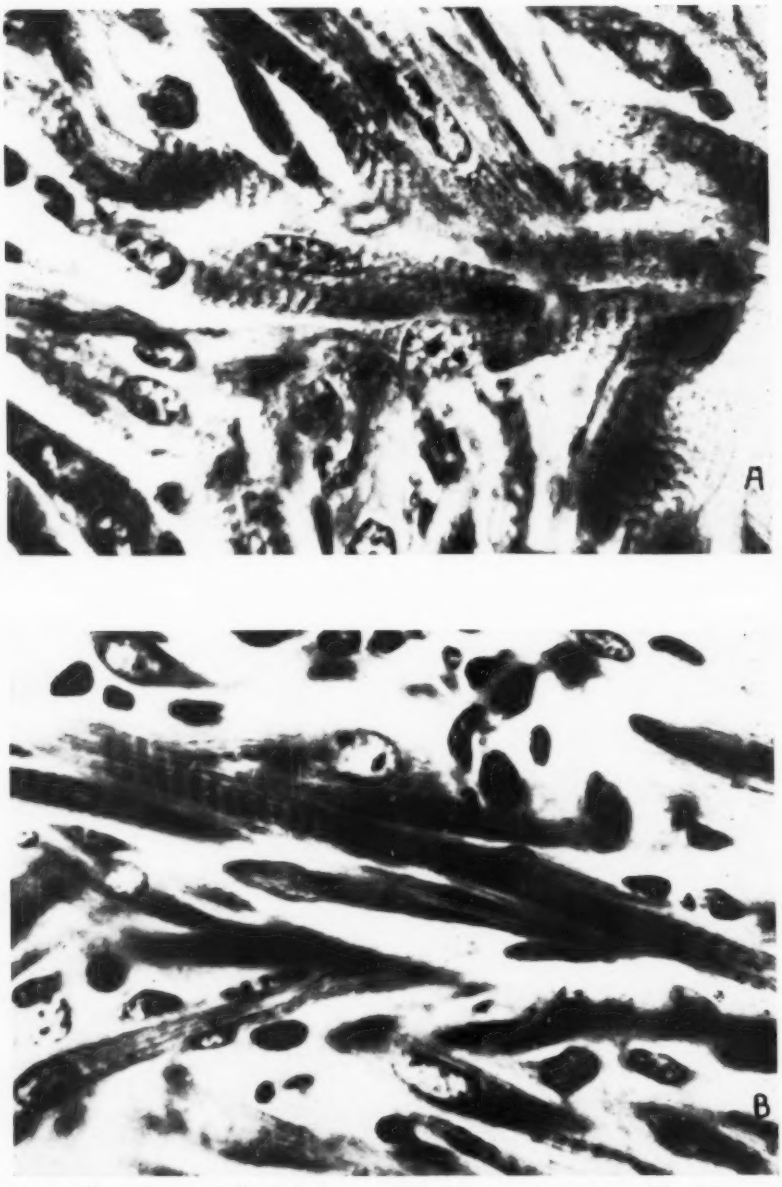

FIGURE 2.—*A*, Photomicrograph of portion from a tumor of the second transplant. The cells resemble the short feathers of a Barred Rock fowl. They are shorter and thicker than those seen in the original. One such transplant showed no striated fibers at all. Bodian's stain. Approximately × 610. *B*, Microscopic pattern of original tumor. The normal-looking cross disks are shown. Phosphotungstic acid-haematoxylin stain. Approximately × 610.

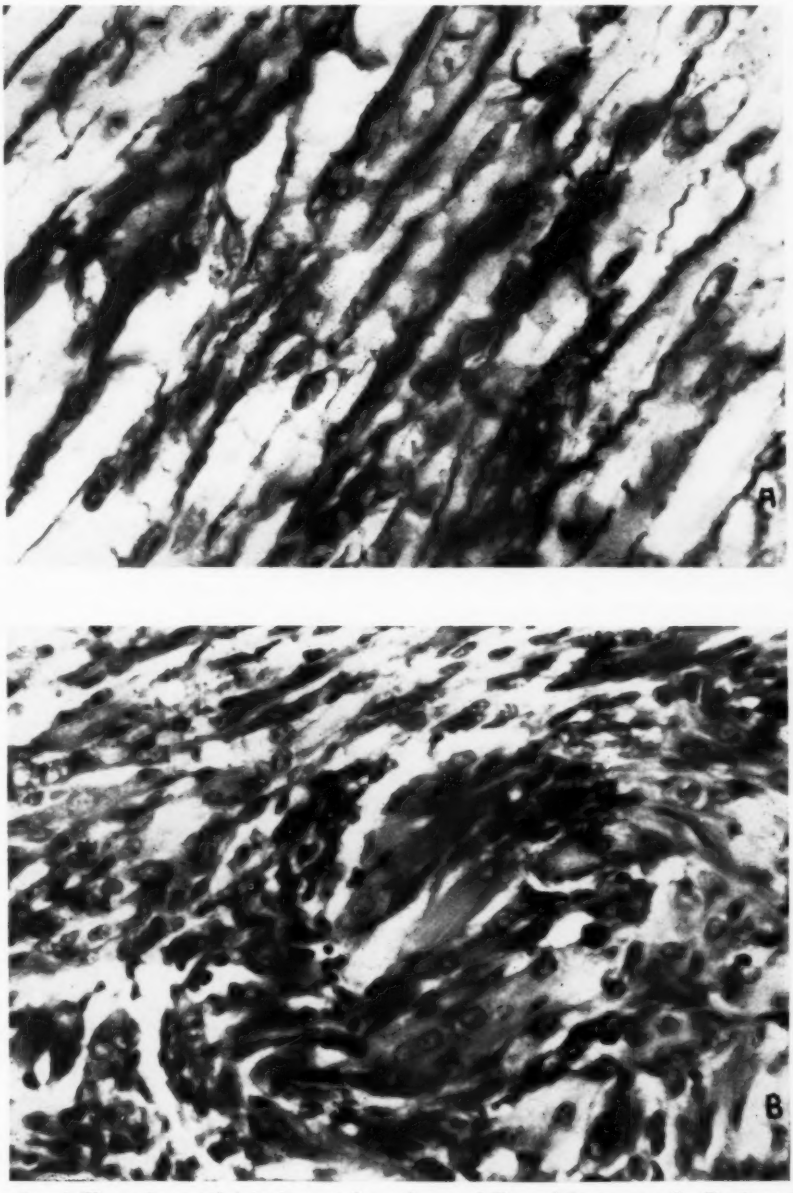

FIGURE 3.—*A*, Photomicrograph bringing out the peri-stromal fibers of the tumor cell. Foot's modified Bielschowsky silver stain. Approximately × 200. *B*, Photomicrograph showing wild arrangement, fasciculation, and polymorphism. Haematoxylin and eosin stain. Approximately × 200.

cause grossly and microscopically it appeared to be in contraction and yet no nerve endings could be found to be present.

I have been unable to find any report of a true spontaneous rhabdomyosarcoma or rhabdomyoblastoma occurring in either mice or rats (*4, 5, 6, 7, 8*). Feldman (*8*) in 1932 was able to find but 12 cases of these tumors in all classes of lower animals. Those he did report were in horses, sheep, hogs, cattle, and chickens. By 1940 there were between 45 and 50 cases of rhabdomyocarcoma of the skeletal muscle reported in man (*3*). More than half of these were reported in the 4 years prior to that time. Fibrosarcomas (*4, 9*) are the common ones in mice mentioned in the literature. Descriptions and photographs of some of these suggest that they may have been true rhabdomyoblastoma of insufficient differentiation to cause diagnostic attraction. Embryonic and traumatic origins have been thought to be significant in their etiology. The former idea fits in well with their rarity, the structures of the body wall having an extremely early differentiation (*10*). Keith (*11*) believes that no new fibrils are formed in skeletal muscles after birth.

There has been considerable difficulty in correlating the histologic behavior of these tumors with their clinical behavior (*12*). The transplants of this tumor showed great variability in histologic structure. Some were chiefly of adult type, similar to the original tumor, others were almost totally rhabdomyoblastomic in form; yet no metastases were ever observed in either form. The constancy of the blood-filled cysts suggests that they are an integral part of the tumor. The tumor most closely resembling this one is the one pictured by Gordon-Taylor (*13*) from a human being. The tumor under study is being carried in transplant in order to ascertain its growth aspects and its enzymatic activities.

SUMMARY

A spontaneous, transplantable, mature type rhabdomyosarcoma of strain C mice is described for the first time. Histologically, it is similar to such tumors in man, described previously, and in domesticated animals, but contains more adult type cells with intact cross striations and myofibrillae. Many cells appear to be in contraction. No nerve endings can be proved to be present.

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Some Fundamental Aspects of Tumor Development Illustrated by Studies With Ultraviolet Radiation

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INTRODUCTION

Is a tumor cell formed immediately upon exposure to a carcinogenic agent, or must the agent act upon the cell for a considerable length of time or a number of times intermittently? This is an important question to be answered as a first step toward the explanation of carcinogenesis. Each of these possibilities has been suggested at one time or another, and numerous hypotheses have been built upon them.

The principal difficulty encountered in attempting to answer this question experimentally is that the measurement of the time from application of a carcinogen to the appearance of a tumor, which I shall refer to as tumor development time, or t_d,[1] includes the growth of the tumor as well as the period actually required for induction. It is impossible at present to determine when the first tumor cell appears, and hence the end point observed must always be based upon an accumulated mass of cells, i. e., a tumor of a given size. This is true whether the tumor is observed grossly, microscopically, or by an indirect method, let us say for example, a change in the chemistry of the tissue. Thus an important part of the tumor development time, however measured, represents growth of the tumor to a given size.

This paper presents the elements of an analysis of the tumor development time for tumors induced by ultraviolet radiation. It is probable that the same fundamental analysis will apply to other types of carcinogenesis, although various factors may differ in detail. The term "development" is used throughout this paper to include all changes which may ultimately lead to the appearance of a tumor. In this sense, the term does not necessarily imply the actual existence of tumor cells at all stages of development, nor is it synonymous with growth.

EXPERIMENTAL PROCEDURE

Certain new experiments have demonstrated pertinent facts in addition to those described in earlier papers in this journal, and these will be discussed first. Male strain A mice have been used throughout the experiments, and the general conditions and criteria of tumor selection described in previous papers have been maintained (*3*, *4*). In the experiments reported thus far, exposure of the animals to ultraviolet radiation was repeated at regular intervals up to the time the tumors reached the standard size for selection. In the present experiments, the schedule of exposures was interrupted for a certain period, or discontinued completely after a given time.

[1] *This term is used herein to replace induction time as used in earlier papers* (1, 2). *The reasons for abandoning the use of induction time in this sense, and of latent period as used by some authors will become obvious in the course of the paper.*

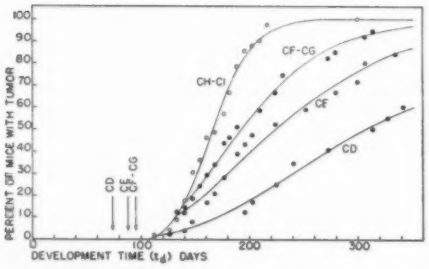

FIGURE 1.—Effect of discontinuing exposures on development time, t_d, for tumors induced by ultraviolet radiation. Group CH–CI received regular exposures up to the time the tumors appeared. For the other groups, the exposures were discontinued at the time indicated by the appropriately labeled vertical arrows.

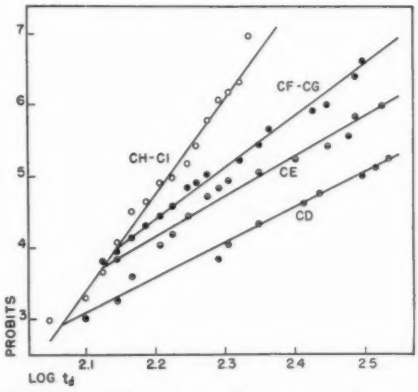

FIGURE 2.—Effect of discontinuing exposures on development time for tumors induced by ultraviolet radiation. Data plotted on probit, log t_d graph.

EFFECT OF DISCONTINUING EXPOSURES

Figure 1 illustrates an experiment in which one group of animals (CH–CI) was exposed in the usual fashion until the tumors appeared, while in three other groups (CD, CE, and CF–CG) the exposures were discontinued after varying times as indicated in the figure. In these three groups tumors continued to appear, although with few exceptions there was no gross evidence of their existence at the time the exposures were discontinued.

In figure 2 the logarithm of the individual development time is plotted against probits (integrated probability units) as in previous studies (*1*). The straight lines obtained indicate that within each group the logarithms of the individual development times are normally distributed. At the time the exposures were discontinued, tumor development must have reached different points in the different individuals. Since development of the tumors continues without the accelerating effect of further exposures, it might be expected to progress most rapidly in those individuals in which tumor development is most advanced. From inspection of figure 2 it appears that tumor development had reached such a point in some of the animals that further exposures would have had no appreciable accelerating effect, whereas in others this acceleration was missed to a degree corresponding to the stage of development reached when the exposures were discontinued.

In table 1, data represented in figures 1 and 2 are compared with those from other experiments, the "average" development time, t'_{50},[2] being taken as the basis of

TABLE 1.—*Effect on tumor development time of discontinuing exposures to ultraviolet radiation*

Group	Number of animals	Dose per exposure (Ergs/cm.^{2} x 10^3)	Exposures per week	Exposure period	Number of exposures	"Average" development time (t'_{50})
			Number	*Days*		*Days*
CH-CI [1]	45	1.8	5	166	117	166
CG, CF	41	1.8	5	95	68	193
CE	25	1.8	5	88	63	225
CD	25	1.8	5	74	53	306
CM-CN [1]	52	5.0	5	150	106	150
CL	26	5.0	5	87	62	192
B-F [2]	49	3.3	5	153	110	153
K	26	8.6	5	12	10	[3] >365
L	26	86.0	.....	1	1	[3] >365

[1] Exposures continued throughout.
[2] Best data for comparison with K and L.
[3] No animals developed tumors within 1 year.

[2] *t_d represents the individual development time. For purposes of discussion t_{50} may be regarded as the "average" t_d for a given group; its exact meaning was explained previously* (1).

comparison. In group L which received a single exposure at very high dosage and in group K which received 10 exposures at high dosage, none of the animals developed tumors within the subsequent year. Histopathologic examination made at the end of that time showed evidence of extensive fibrosis but no suggestion of tumor development. This might be taken as evidence that a minimum number of exposures (over 10) is required in order to initiate the tumor process. However, a simple extrapolation shows that this is not necessarily true. In figure 3, values of t'_{50} for groups CG–CF, CE, and CD, which received the same dosage per exposure, are plotted against the corresponding numbers of exposures received. The three points fall on a straight line, and if this is continued to 10 exposures we find that the corresponding t'_{50} value is 645 days. Obviously, considerable error may enter this extrapolation, for the straight-line relationship may not hold; but in any case it seems highly improbable that 50 percent of the animals exposed only 10 times would develop tumors within the normal life span even if tumor development were already initiated at the end of the exposures. The line t_4 in figure 3 is drawn to represent the corresponding curve for 4-percent incidence. The 10-exposure point falls at 470 days, indicating that 1 animal in a random group of 25 would develop a tumor at that time. This line has been drawn by using the average standard deviation obtained for a number of experiments in which the exposures were continued until the appearance of tumors (*1*) and probably indicates later appearance of tumors than would obtain in the present case.[3] Nevertheless, it appears that even if the tumor process

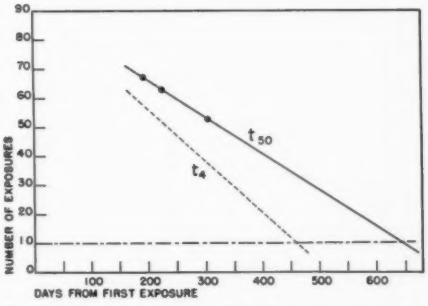

FIGURE 3.—Effect of number of exposures on development time. t_d. $t_{50}=t_d$ for 50-percent incidence; $t_4=t_d$ for 4-percent incidence.

had been initiated and if there had been no recovery, the chance that 1 animal of our group of 26 would develop a tumor within 1 year after the exposure is remote.

These experiments show that continued repeated exposures to ultraviolet radiation accelerate the development of tumors, and that their discontinuance may result in a delay in the appearance of the tumor depending upon the stage of its development at the time the exposures are discontinued. They also indicate that a single exposure, or even a limited number of exposures, to ultraviolet radiation does not cause tumors to develop within the lifetime of the mouse. This does not rule out the possibility, however, that tumor development is actually initiated after a few exposures, but that it progresses too slowly for the tumor to appear in the animal's lifetime; the experiments described in the following section indicate that this is the case.

EFFECT OF INTERRUPTING EXPOSURES FOR A LIMITED PERIOD

In these experiments the exposures to ultraviolet radiation were discontinued after a certain period but were resumed again after an interim of rest. The results of two sets of experiments are summarized in table 2. Groups BP and BM received exposures at regular intervals until tumors

[3] *Note that the slope of the lines in figure 2 is less when the exposures are discontinued and that this slope is the reciprocal of the standard deviation* (1).

appeared, whereas in each of the other groups the exposures were discontinued for approximately 1 month, then resumed, and were continued until tumors appeared. During the first month all groups received exposures (period 1); groups BN and BK received no exposures during the second month (period 2), while groups BO and BL received no exposures during the third month (period 3). The weekly dosage was the same during all periods (12.3×10^7 ergs per square centimeter per week). After period 1 this was divided into five doses a week for all groups,[4] but during the first period groups BP, BO, and BN received exposures every other day, while groups BM, BK, and BL received exposures only every fourth day. Because of this change in schedule of exposures the incidence curves are somewhat distorted, but this should not vitiate the t'_{50} values reported in the table.

TABLE 2.—*Effect on tumor development of interrupting exposures to ultraviolet radiation for a limited period.*

Group[1]	Period 1		Period 2		Period 3		"Average" development time t'_{50}	Increase in t'_{50}
	Length	Exposures	Length	Exposures	Length	Exposures		
	Days	*Number*	*Days*	*Number*	*Days*	*Number*	*Days*	*Days*
BP	39	20	29	19	27	19	174	.....
BN	39	20	29	.....	27	19	187	13
BO	39	20	29	19	27	.....	178	4
BM	36	10	31	19	27	19	176	.....
BK	36	10	31	.....	27	19	211	35
BL	36	10	31	19	27	.....	200	24

[1] All groups consisted of 26 animals.

In all groups that enjoyed an interim without exposures (BN, BO, BK, BL), the tumors appeared later than in those groups that received continued exposures (BP, BM), as might have been predicted on the basis of the experiments described in the preceding section.

[4] *The dosage was in all cases above that which produces maximal effect* (2).

The most striking feature of these experiments is the demonstration that tumor development is initiated after a few exposures, even as few as 10. This may be seen by comparison of groups BK and BM in table 2. Group BK received 10 exposures followed by an interim of 31 days, and tumors appeared in a much shorter time after the exposures were resumed than would have been required had there been no previous exposures as in the case of group BM. The t'_{50} for group BK was 211 days, 67 days of which were taken up by the first exposure period (36 days) and the rest period (31 days). Thus only 144 days were required for tumor development after the rest period, whereas 176 days were required for comparable tumor development in group BM. This can only mean that the first 10 exposures brought about some degree of tumor development, as defined earlier in this paper (p. 569), a conclusion which may appear at first to be contrary to the finding that 10 exposures alone did not produce tumors. (See group K, table 1.) However, in group BK the delay in appearpearance of tumors (represented by the difference in t'_{50} for BK and BM) is 35 days, which is approximately equal to the interim without exposures (31 days); and this suggests that the degree of tumor development did not alter appreciably during that interim. It would appear that tumor development was slow or was even regressing at this stage, so there would be no reason to expect that tumors would develop within the lifetime of the animals unless development were accelerated by further exposures.

The other experiments in which the exposures were interrupted (BL, BN, and BO) indicate that not only has tumor development been initiated before the interim without exposure but that it has progressed during that interim. This is

obvious, since the increase in t'_{50} is less than the interim in all cases, including group BN which received only 20 exposures before the rest interim. It appears that tumor development progresses without further application of the carcinogenic agent after as few as 20 exposures. The differences between t'_{50} and its expected value if no tumor development occurred during the rest interim reach statistical significance in only 1 of these cases, since it may be estimated from previous studies that a difference of t'_{50} of 20 to 25 days might be expected to occur once in 20 times owing to chance alone (*1, pp. 87–88*). However, the fact that the differences occur in the 4 cases in the order that would be expected under the respective experimental conditions if tumor development continued during the interim, leads to the conclusion that this is the case. (See p. 576.)

DISCUSSION

The experiments just described indicate that tumor development, using this term as heretofore defined (p. 569), is already under way after a few exposures to ultraviolet radiation. They tell us nothing, however, as to the nature of the developmental changes beyond the fact that development may progress in the absence of the exciting agent. For example, this early development might be a manifestation of changes which precede the formation of tumor cells, or it might represent the existence of proliferating tumor cells. Both of these possibilities are considered in the following attempt to analyze the components of development time, in which inherent difficulties confronting such an analysis are pointed out.

In attempting such an analysis one can only begin with an idealized picture and subsequently examine it to find out how well it fits the experimental data, or to what extent it must be modified. Several possibilities are explored in the following pages, but all require a basic assumption which seems essential to any hypothesis regarding tumor development. This basic assumption is that once a tumor cell comes into existence, it reproduces itself at intervals by equal division, and that the growth of the tumor follows this process quantitatively. Such an ideal condition is to be expected only if there is no interaction of cells, as would be the case if they were infinitely dispersed. Obviously, this condition does not obtain, but such growth relationships may be approached at some time in tumor development. The idealized growth process would be described for a single cell division by the equation:

$$\frac{\Delta N}{\Delta t} \propto N \qquad (1)$$

where N is the total number of cells at given time t, Δt the time required for a complete cell-division cycle, and ΔN the increase in number of cells brought about by this division.

Actually, cell division would be occurring at all times in a tumor made up of a large number of cells, and hence growth of the tumor would manifest itself as a continuous process which would be described by the equation:

$$\frac{dN}{dt} = \mathrm{k}N \qquad (2)$$

where k is a constant.

By integration between appropriate limits equation 2 becomes

$$N = N_o e^{kt} \qquad (3)$$

in which N_o is the number of cells at zero time, which may be arbitrarily selected, and e is the base of natural logarithms. This is the basic equation for exponential growth.

Equation (3) may be written in logarithmic form with k replaced by k of which the value is chosen so as to take care of the

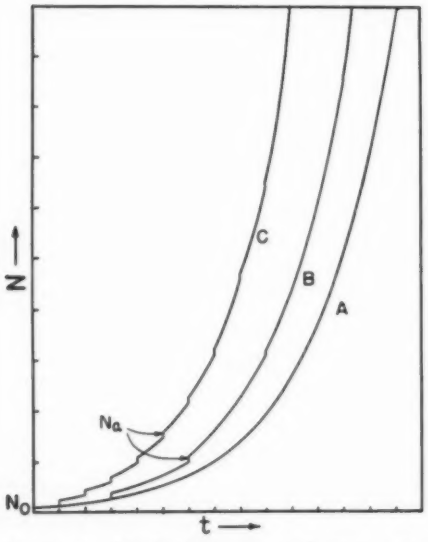

FIGURE 4.—Idealized tumor-growth curves, numerical ordinates. For explanation, see text.

change from natural to Briggsian logarithms:

$$\log N - \log N_o = kt \qquad (4)$$

This is a more useful form for many purposes.

The curves labeled A in figures 4 and 5 are described by this equation; in figure 4 numerical ordinates are used and in figure 5, logarithmic ordinates. The latter graph is useful because it yields a straight line, and it will be frequently employed to illustrate the following discussion.

It is possible to make estimates of the rate of growth of these tumors in their later stages and of the number of cells they contain at the time of selection and to test the application of this idealized model by employing the estimated values. Such estimates have been made on selected tumors which were as nearly spheres or spheroids as could be found. Prolate spheroids are most common, but spheres and oblate spheroids occur. Measurements were made along three axes of the tumor corresponding as nearly as possible to the three principal axes of the ear. These measurements yield three values which may be assumed to be three diameters of a sphere or spheroid, a, b, and c, and the volume of the tumor may then be estimated using the formula $4/3\pi \frac{abc}{8}$. Deviation in shape from true spheres or spheroids or the inclusion of normal tissue should give high values for the volume of the tumor; the latter factor is of importance only in small tumors. Measurements were made at intervals of a few days in order to follow tumor growth. The fact that the measurements were made each time on approximately the same axes should make the relative error in measuring tumor growth less than that involved in estimating the actual volume, so long as the shape of the tumor does not alter radically between measurements. At best these estimates are rough, but they yield growth curves which suggest that equation

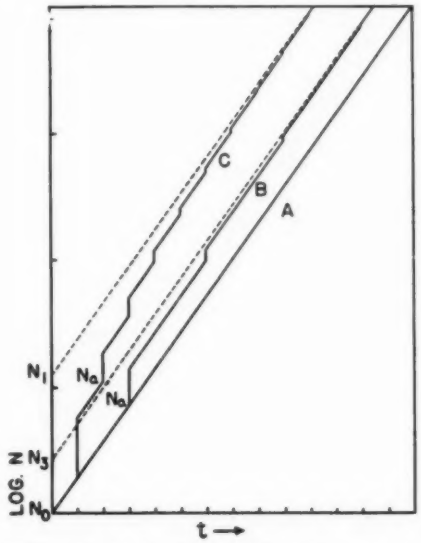

FIGURE 5.—Idealized tumor-growth curves, logarithmic ordinates. For explanation, see text.

(4) is approximately followed at the time of selection but that the relative growth rate falls off as the tumors reach large size. They indicate a high average rate of growth at about the time we have chosen for selection, showing that the end point for our measurements of tumor induction time (*t*) is sharp. At this point the tumor has a volume of about 100 mm.^{3} and on the average is doubling its size about every 4 or 5 days. If homogeneity of the tumor may be assumed, the same numerical value of k is obtained if volumes are substituted for numbers of cells in equation (4). Estimates from measurements on 22 tumors yielded an average value of 0.065 for k in equation (4), with a standard deviation of 0.0375.

The number of cells in a sarcoma having the volume of the tumors at selection time and composed of closely packed cells may be estimated as roughly 10^8 cells. The number must vary considerably among tumors, so that this estimate is very rough at best, but it will serve as a basis for our calculations and will be designated as N_s, or number of cells at the time of selection.

Induction Period Followed by Unrestricted Cell Proliferation

If in equation (4) N_s is substituted for N, N_o is taken as 1, and k is given the value 0.065, t has the value 123. This means that a tumor growing continuously at the average rate which these tumors display at selection time would develop from a single cell in 123 days. Reference to table 1 shows that the time to t'_{50} following the cessation of exposures is greater than this in some instances; for example, exposure of group CD ceased after 74 days and t'_{50} was reached 240 days later. It is obvious then that if tumors grew without restriction at the rate which they display at selection time, it would be necessary to assume that less than one tumor cell was formed at the end of the exposure period in this case. Since the existence of growing cells at the end of 68 days (group BO) is indicated by the data in table 2, which suggest their existence much earlier, the absurdity of this picture is enhanced. The estimate just made depends upon two values N_s and k, neither of which is accurately determined, but the substitution of any other set of reasonable values for these terms will not permit the data of tables 1 and 2 to be rationalized by means of equation (4).

Thus, the simple assumption that tumor cells are formed only after a given period of repeated exposures to ultraviolet radiation (induction period) and thereafter grow without let or hindrance, cannot be reconciled with the experimental facts.

This situation is somewhat altered if the assumption is made that the developmental change which goes on during the induction period is not only the accumulative result of repeated exposures to ultraviolet radiation, but is self-proliferative, i. e., that it continues to progress after the withdrawal of the exciting agent. In this case, proliferation of tumor cells might be regarded as a terminal process occurring only as a sequel to another self-proliferative process. Such a mechanism would be too complicated for a simple analysis based on the existing experimental evidence, but in certain quantitative aspects would resemble that discussed later.

Acceleration of Tumor Development by Repeated Addition of Tumor Cells

The experiments in table 1 show that a single exposure to ultraviolet radiation does not result in the appearance of tumors within the lifetime of the animals. In addition, they indicate that repeated exposures serve to accelerate the development of the tumors. Let us consider the type

of growth curve to be expected if growth goes on exponentially between exposures, as indicated by equation (4), but is accelerated by the addition of new tumor cells at each exposure. To do this, the simple assumption may be made that a given number of cells N_a is produced at the first exposure and that an equal number is added at each subsequent exposure. The consequences are illustrated in figures 4 and 5, where in each case curve A represents growth from N_o cells without further addition of cells, whereas curves B and C show the effect of adding N_a cells at regular intervals.

The fact that tumors cannot be detected in the early stages at which our experiments indicate tumor development to be going on provides no valid objection to this hypothesis, for no method has yet been devised which permits identification of a tumor cell that is not in close association with a considerable number of other tumor cells. Many such cells might be scattered through the normal tissue without being detected, the identification of microscopic tumors being possible only after these cells have proliferated to a sufficient extent to form aggregates that display a characteristic histologic appearance.

Several points may be noted at which this analysis fits, qualitatively, with experimental findings. The accelerating effect of the earlier exposures would be much greater, proportionately, than that of the later exposures, with the result that after a certain time further exposures have little or no effect on the rate of tumor growth (curve B, figs. 4 and 5). This seems to be the case experimentally, for the data in table 1 suggest that the later the exposures are discontinued the less is the relative delay in the appearance of tumors. When the exposures are applied at less frequent intervals, the accelerating effect would be relatively less (curves B and C, figs. 4 and 5). This result agrees with what has been found experimentally (*2*). Both of these findings help in explaining the data of table 2. In both experiments described therein the retardation of the development time was greater when the interim without exposure occurred in the second month than when it occurred during the third month. Such a difference would be anticipated since the accelerating effect of the exposures would be greater in the earlier period, and correspondingly their omission would be missed to a greater extent. In the second series of experiments, the exposures were given less frequently during the first month than in the first series, and hence the number of tumor cells should have been less at the end of that time. This is equivalent to an earlier position on the growth curve, and the fact that the rest interims caused greater delay of tumor appearance in the second series of experiments than in the first fits with the analysis.

The fact that a single exposure does not induce tumors makes it necessary to assume that it is impossible to produce more than a limited number of tumor cells at one exposure, and this fits with the experimental finding that for a given exposure schedule there is a minimum development time which cannot be decreased by increasing the dose of radiation (*2*). Above a certain dose, therefore, the growth curves would be identical as long as the schedule of exposures is maintained. The development time is greater when the exposures are given at less frequent intervals (*2*), an experimental finding which also fits with this analysis.

The effect of different dosage schedules on the ratio of carcinomas to sarcomas which was pointed out by Grady, Blum, and Kirby-Smith (*6*) may be explained on this same basis. Dose, per se, does not affect this ratio, but it increases slightly

with t_d, and is markedly decreased by increasing the interval between exposures If it is assumed that the carcinomas grow somewhat more slowly than the sarcomas, the increase in the ratio with t_d may be explained because the longer we wait the greater is the chance that the carcinoma will reach detectable size. Moreover, the effect of repeated exposures would be greater in the case of the more slowly growing tumor, so that more frequent exposures should tend to increase the incidence of detectable carcinomas.

The hypothesis may be expressed quantitatively by a modified form of equation (4). The number of cells added by exposure is represented at any given time t by $N_a\frac{t}{E}$ where E is the interval between exposures. As will be seen by reference to figure 5, the result of adding new cells by exposure is equivalent to increasing the value of N_0 at zero time. Note that in the figure, N_1 represents the apparent value of N_0 after N_a has been added 10 times at unit intervals of time, while N_3 represents the apparent value of N_0 after N_a has been added 4 times at intervals of 3 time units. Thus, we might substitute $N_a\frac{t}{E}$ for N_0 in equation (4). But N_a is actually equal to N_0 in equation (4) since it represents the number of cells formed after 1 exposure, i. e., at zero time. Hence we may write the equation for tumor growth accelerated by the addition of a given increment of cells at regular intervals, as

$$\log N-\log\left(N_0\frac{t}{E}\right)=kt$$

or (5)

$$\log N-\log N_0-\log\frac{t}{E}=kt$$

In its simple form the hypothesis displays the same weakness as that of an induction period followed by unrestricted growth, which was discussed in the preceding section. By substituting values for E and t from any of our experiments (*1*) and using the same values of N_s and k as heretofore, the value of N_0 obtained from equation (5) is always a small fraction, i. e., less than one cell—an obvious absurdity. That the consequences of the two hypotheses are parallel in this respect is seen from a consideration of figure 5, which makes it apparent that if cell proliferation goes on with a constant value of k, the addition of increments of cells has virtually no effect after the first few additions unless k is extremely small.

If the growth of tumors were for some reason much slower in early than in later stages, the above-mentioned discrepancy might be explained. Some support is given to this concept by the following calculations. Data are available for three schedules of exposure, 1, 5, and 7 per week. As pointed out previously (*2*, *fig. 1*), t'_{50} does not decrease with increase in dose above a certain critical dose value, the minimum for t'_{50} being different for each schedule of exposures. This fact seems best explained by the assumption that there is a maximum number of tumor cells that can be formed at a single exposure; for if this is the case, the critical dose level N_0 in equation (5) has the same value for all exposure schedules. On the basis of this assumption, simultaneous equations having the form of (5) may be set up using the minimum value of t'_{50} for the three schedules, with the corresponding values of E, and solved for k. The resulting values of k calculated for the three pairs of equations are very nearly the same, approximately 0.006. Considering the inaccuracies of the data, this is rather remarkable agreement which can hardly be attributed to chance and which suggests that the present hypothesis

is in essence valid. The value of k obtained in this way corresponds to cell division every 50 days, i. e., the tumor volume should double in that time. This is about one-tenth the average growth rate found at selection time. (See p. 575.) Thus the value of k obtained from this calculation cannot represent a true index of cell proliferation rate throughout the development time. If it has any significance at all, it is as an "average" index rather than an actual measure of the rate of cell division at any given time. For such an average rate index to obtain, cell-division rate must be slower than once in 50 days in its early stages, for in later stages it is much more rapid.

Control of Tumor Growth

The assumption that tumor growth is much slower in early than in late stages permits the rationalization of the hypothesis that tumor development results from continuous growth plus the addition of fresh tumor cells, with the existing data. It also permits a certain degree of rationalization of the hypothesis of an induction period followed by tumor growth; but only if the induction period is assumed to be short, or if the induction process is assumed to be capable of progressing in the absence of the exciting agent. A plausible explanation for such a progressive change in relative growth rate is that normal tissue exerts a controlling influence on tumor development which becomes less as the tumor grows, until finally the tumor virtually escapes from this influence and has almost unrestricted growth for a time. Sooner or later, of course, the growth of the tumor must again be restricted by nutritional or other factors, since continued exponential increase with time is impossible in such a system.

The concept of a controlling influence of the normal tissue is an attractive one, which fits with much that is known of developmental morphology. One may find many examples of such an effect in the literature of experimental embryology (*5*). The concept may be given a semi-quantitative basis by assuming that the controlling effect is proportional to the amount of normal tissue and hence has a constant value. With an increase in the tumor mass while the normal tissue mass remains approximately constant, the controlling influence of the normal tissue must diminish progressively in relative importance. Thus the tumor may grow slowly at first but eventually may escape from the restraining influence. The normal tissue mass may be taken to be that immediately involved at the site of origin of the tumor, or, if it seems desirable to include the possibility of hormonal effects, it may be taken to be the whole animal.

Control by the normal tissue might be accomplished in more than one way. For example, the rate of cell division of the tumor cells might be inhibited by the normal tissue so that the rate actually changes with time as a function of the ratio of tumor to normal tissue. As an alternative one may postulate that the controlling action of the normal tissue depends upon the destruction of tumor cells at a more or less constant rate. This may well be a factor since in those experiments in which the exposures were discontinued so early that tumors did not develop (table 1), no evidence of microscopic tumors was disclosed by histopathologic examination 1 year later; the most distinctive feature at that time was fibrosis of the subepidermal tissues, the epidermis appearing virtually normal.

It may be possible, eventually, to place such a hypothesis on a strictly qualitative basis. Shock and Morales (*7*) have re-

cently succeeded in describing a number of types of organismal growth by the use of the differential equation they formulate; and if adequate experimental data can be obtained, it may be possible to apply the same treatment to tumor growth. These authors find that growth curves can be expressed in terms of two factors, the first of which, ϕ, is the intrinsic ability of cells to proliferate. It is assumed that the rate of cell increase is always proportional to the number of cells present at any instant, but that the "constant" of proportionality may vary with time. This would be the case if the rate of cell division were proportional to the ratio of tumor to normal tissue and therefore would vary with time as this ratio varies. Their second factor, θ, affects growth by interaction between the cells. The interaction between normal and tumor tissue resulting in the destruction of cells might be included in this factor. Further investigations, including experiments of the type of those described earlier in this paper, may provide the data necessary for analysis following these concepts; but until such data are obtained, it can be carried no further.

Factors Determining Development Time

Whatever mechanism may eventually be assigned to tumor development, it is certain that during the period of growth the rate of tumor-cell proliferation is proportional at any instant to the number of tumor cells present. This must be true whether or not the period of growth is preceded by a period of induction during which no growth takes place. That this proportionality between rate of increase and existing number of cells may change with time does not alter the basic argument. Hence equation (3) may be used as an idealized model for certain purposes of discussion, as long as we realize its limitations in describing actual tumor growth. Equation (5) which applies to experiments with repeated dosage such as are required for induction of tumors by ultraviolet radiation is a special form of (3).

Equation (3) illustrates a very important point often lost sight of in quantitative tumor investigations, that is, that the time required for growth of a tumor to a given size depends not only upon the rate of cell proliferation, the index of which in these equations is k, but upon the number of cells present at the beginning of growth, N_o. Thus we must recognize at least two fundamental factors in tumor development which I shall name proliferation and susceptibility. The proliferation factor is measured by k. Susceptibility, on the other hand, is represented by the number of tumor cells formed by the carcinogenic agent at the beginning of the growth period, whether this be reckoned as occurring immediately after the application of a carcinogenic agent, or after an induction period during which growth does not occur. This number is, of course, N_o, which hence may be taken as a measure of susceptibility.

However, if only these two factors were operative, the development time would be much shorter than observed, and hence it is necessary to admit the existence of at least one additional factor. If we employ x to describe such a factor, we may write

$$t_d = f_1(p), f_2(s), f_3(x) \qquad (6)$$

where t_d is the development time, i. e., the time between the initiation of the carcinogenic action and the appearance of a tumor of a given size; p is the rate of cell proliferation, itself a function of k in equation (3); s is the susceptibility to carcinogenic action, which expresses itself as N_o.

If there is control by the normal tissues, as suggested above, it is necessary to introduce a factor c which operates to modify the proportionality between cell increase and number of existing cells. (See p. 578.) This factor may replace x, so that equation (6) becomes:

$$t_d = f_1(p), f_2(s), f_3(c) \qquad (7)$$

It is obvious that factors p, s, and c operate simultaneously to determine the value of t_d and hence cannot be separated in time. If an induction period exists during which no growth or other self-proliferative change takes place, the equation should be written

$$t_d = f_1(p), f_2(s), f_3(c) + t_i \qquad (8)$$

where t_i is the induction period. Such a possibility was considered in an earlier paper (*2*), where it was pointed out that if t_i exists it is probably proportional to the period of growth and thus not separable in time from the other factors which form the first member of the equation. Thus, if equation (7) is taken as a more general form of (6), it must be understood that proportionality between t_i and t_d is probably established in some way not indicated. There is no conclusive evidence for the existence of an induction period of the nature of t_i, and the foregoing discussion must indicate the difficulties involved in establishing such evidence.

No matter what hypothesis is accepted, factors p and s and at least one additional factor must operate in determining the development time when tumors are induced by ultraviolet radiation. It seems probable that the same is true for other carcinogenic processes, and hence equation (*6*) may be taken as a general equation for t_d. In such a general equation x may be the expression of more than one factor. This fits, for example, with the concept of Brues, Weiner, and Andervont (*8*), who find a certain correlation between the development time (their latent period) and the mitotic index, which they use to estimate tumor growth. They recognize the importance of the initial number of cells (our susceptibility factor), but their evidence does not test the existence of an x factor. The correlation that they point out is in complete accord with the foregoing analysis, but the existence of the susceptibility and x factors, which presumably may vary independently, would make it possible that numerous exceptions to their correlation occur.

Unfortunately, there is no way of separating the factors p, s, and x by existing techniques, and hence we are without quantitative basis for a definitive theory of carcinogenesis. As pointed out at the beginning of this paper, the measurements of the time between the application of the carcinogen and the appearance of a tumor of a given size (t_d) is the basis for all measurements of carcinogenesis, yet this value must depend upon at least two, in all probability three, simultaneously operative factors. This is no argument against the use of t_d for comparative purposes, so long as we do not err in interpreting the cause of observed difference in this value. For example, if t_d differs significantly for two groups of animals treated in different ways, the difference should not be attributed to, say, different rates of cell proliferation, when it is equally possible that it is caused by differences in susceptibility or x factors, unless there is other evidence to support the thesis offered.

SUMMARY

Experiments on induction of cutaneous tumors in mice by ultraviolet radiation are described, in which repeated exposures to that agent were discontinued after varying periods or were interrupted for limited periods and subsequently resumed. These

experiments show that such exposures accelerate tumor development even in relatively late stages. They also show that changes leading to the appearance of tumors are initiated very early, but that tumors may not occur in the animal's lifetime if the exposures are discontinued too early, even though such changes have been initiated.

Various explanations of these facts are considered. It is pointed out that at least three basic factors must be assumed to operate to determine the time required for appearance of tumors.

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